Platelets in Thrombotic and Non-thrombotic Disorders

Platelets have attracted increasing interest among clinicians and basic scientists over recent years, and are now known to play a part in many physiological and pathological conditions. In fact, platelets are involved in diseases responsible for the majority of disability and death worldwide, including myocardial infarction, stroke, peripheral vascular disease, cancer, and many infections. Platelets are also studied as a model in many areas of neurobiology, pharmacology, biochemistry and molecular biology.

This timely reference provides a comprehensive, detailed, up-to-date resource for clinicians and researchers, covering the structure and function of platelets and their role in pharmacology, pathogenesis and therapeutics. It is organized into sections covering platelet physiology, laboratory and methodological issues, platelet involvement in disease, including hemostatic and non-hemostatic conditions, platelet pharmacology, and treatment in the clinic.

With contributions from leading authorities including some of the founders of modern platelet studies, this is the definitive reference and guide to the diagnosis and therapeutics of diseases involving platelets. Chapters are devised to provide a critical review of the most clinically relevant aspects of the subject, with extensive references and easy-to-read take-home messages. It will be an essential resource for biomedical scientists and clinicians in hematology, vascular medicine, cardiology, thrombosis and related disciplines.

Paolo Gresele is Associate Professor of Internal Medicine, Section of Internal and Cardiovascular Medicine, Department of Internal Medicine, University of Perugia, Italy

Clive Page is Professor of Pharmacology, Division of Pharmacology and Therapeutics and Director of the Sackler Institute of Pulmonary Pharmacology and Therapeutics, Guy's, King's and St Thomas' School of Biomedical Sciences, London

Valentin Fuster is Director of the Zena and Michael A. Wiener Cardiovascular Institute and the Richard Gorlin, M.D./Heart Research Foundation Professor of Cardiology at the Mount Sinai Medical Center and School of Medicine, New York, New York

Jos Vermylen is Professor of Internal Medicine, Centre for Molecular and Vascular Biology, University of Leuven, Belgium

Cambridge Books Online © Cambridge University Press, 2009

Platelets

in Thrombotic and Non-thrombotic Disorders

Pathophysiology, Pharmacology and Therapeutics

Edited by

Paolo Gresele

Section of Internal and Cardiovascular Medicine
Department of Internal Medicine
University of Perugia, Italy

Clive P. Page

Sackler Institute of Pulmonary Pharmacology
King's College London, UK

Valentin Fuster

The Mount Sinai Medical Center and
School of Medicine
New York, USA

and

Jos Vermylen

Centre for Molecular and Vascular Biology
University of Leuven, Belgium

Cambridge Books Online © Cambridge University Press, 2009

CAMBRIDGE UNIVERSITY PRESS
Cambridge, New York, Melbourne, Madrid, Cape Town, Singapore, São Paulo

Cambridge University Press
The Edinburgh Building, Cambridge CB2 2RU, United Kingdom

Published in the United States of America by Cambridge University Press, New York

www.cambridge.org

Information on this title: www.cambridge.org/9780521802611

© Cambridge University Press, 2002

This book is in copyright. Subject to statutory exception and to the provision of relevant collective licensing agreements, no reproduction of any part may take place without the written permission of Cambridge University Press.

First published in print format 2002

ISBN-13 978-0-511-05720-5 eBook (Adobe Reader)

ISBN-13 978-0-521-80261-1 hardback
ISBN-10 0-521-80261-X hardback

Cambridge University Press has no responsibility for the persistence or accuracy of URLs for external or third-party internet websites referred to in this book, and does not guarantee that any content on such websites is, or will remain, accurate or appropriate.

Every effort has been made in preparing this book to provide accurate and up-to-date information which is in accord with accepted standards and practice at the time of publication. Nevertheless, the authors, editors and publishers can make no warranties that the information contained herein is totally free from error, not least because clinical standards are constantly changing through research and regulation. The authors, editors and publisher therefore disclaim all liability for direct or consequential damages resulting from the use of material contained in this book. Readers are strongly advised to pay careful attention to information provided by the manufacturer of any drugs or equipment that they plan to use.

Cambridge Books Online © Cambridge University Press, 2009

Contents

List of contributors ix
Editors' preface xxi

PART I PHYSIOLOGY

1 **History of platelets** 3
J. Fraser Mustard, Raelene L. Kinlough-Rathbone and Marian A. Packham

2 **Production of platelets** 25
Arnaud Drouin and Elisabeth Cramer

3 **Morphology and ultrastructure of platelets** 41
James G. White

4 **Platelet heterogeneity: physiology and pathological consequences** 70
Anthony Mathur and John F. Martin

5 **Platelet membrane proteins as adhesion receptors** 80
Yoshiaki Tomiyama, Masamichi Shiraga and Sanford J. Shattil

6 **Dynamics of the platelet cytoskeleton** 93
Kurt L. Barkalow, Hervé Falet and John Hartwig

7 **Platelet organelles** 104
Francine Rendu and Brigitte Brohard-Bohn

8 **Platelet receptors for thrombin** 113
Lawrence F. Brass, Marina Molino, Peter J. O'Brien and Mark Kahn

9 **Platelet receptors: ADP** 127
Christian Gachet and Jean-Pierre Cazenave

10 **Platelet receptors: prostanoids** 140
Roberta Vezza and Garret A. FitzGerald

Cambridge Books Online © Cambridge University Press, 2009

11 **Platelet receptors: collagen** 158
Pia R-M. Siljander and Richard W. Farndale

12 **Platelet receptors: von Willebrand factor** 179
Jerry Ware and Zaverio M. Ruggeri

13 **Platelet receptors: fibrinogen** 188
Edward F. Plow, Thomas A. Haas and Tatiana V. Byzova

14 **Platelet signalling: GTP-binding proteins** 204
Jan-Willem N. Akkerman

15 **Platelet phospholipases A_2** 221
Michael H. Gelb, Carine M. Mounier, Ying Hefner and Steve P. Watson

16 **Roles of phospholipase C and phospholipase D in receptor-mediated platelet activation** 238
Mauro Torti and Eduardo G. Lapetina

17 **Platelet signalling: calcium** 260
Juan A. Rosado and Stewart O. Sage

18 **Platelet signalling: protein kinase C** 272
Charles S. Abrams and Marcelo G. Kazanietz

19 **Platelet signalling: tyrosine kinases** 281
Sylviane Levy-Toledano

20 **Platelet signalling: cAMP and cGMP** 290
James L. Daniel, Barrie Ashby and Fabio M. Pulcinelli

21 **Platelet adhesion** 304
Philip G. de Groot and Jan J. Sixma

22 **The platelet shape change** 319
Adrian R.L. Gear and Renata K. Polanowsa-Grabowska

23 **Aggregation** 338
Marian A. Packham, Margaret L. Rand and Raelene L. Kinlough-Rathbone

24 **Amplification loops: release reaction** 357
Marc F. Hoylaerts

25 **Amplification loops: thromboxane generation** 369
Paola Patrignani and Maria G. Sciulli

26 **Platelet procoagulant activities: the amplification loops between platelets and the plasmatic clotting system** 381
H. Coenraad Hemker

27 **Platelets and chemotaxis** 393
Stefania Momi and Paolo Gresele

28 **Platelet–leukocyte interactions relevant to vascular damage and thrombosis** 412
Chiara Cerletti, Virgilio Evangelista, Roberto Lorenzet and Giovanni de Gaetano

29 **Vascular control of platelet function** 432
Scott Willoughby and Joseph Loscalzo

PART II METHODOLOGY

30 **In vitro assays for evaluating platelet function** 459
Perumal Thiagarajan and Kenneth K. Wu

31 **Monitoring antiplatelet therapy** 471
Dermot Cox and Desmond Fitzgerald

32 **Flow cytometric analysis of platelet function** 485
Alan D. Michelson, Marc R. Barnard, Lori A. Krueger, A.L. Frelinger III and Mark I. Furman

33 **Animal models of platelet-dependent thrombosis** 499
Paolo Golino and Francesco Loffredo

PART III PATHOLOGY

A **Hemostasis**

34 **Hereditary thrombocytopenias** 515
Chris Van Geet, Kathleen Freson, Rita Devos and Jos Vermylen

35 **Thrombocytopenias due to bone marrow disorders** 528
Beng H. Chong and Colin Chesterman

36 **Immune-mediated thrombocytopenia** 542
Kathryn E. Webert and John G. Kelton

37 **Thrombocytopenia in childhood** 556
Cécile Kaplan, M. Dreyfus, V. Proulle and G. Tchernia

38 **Alloimmune thrombocytopenia** 569
E. Anders Kolb and James B. Bussel

39 **Drug-induced and drug-dependent immune thrombocytopenias** 584
Andreas Greinacher, Petra Eichler and Norbet Lubenow

40 **Thrombotic thrombocytopenic purpura and hemolytic uremic syndrome** 610
Miha Furlan

Cambridge Books Online © Cambridge University Press, 2009

41 **Thrombocytosis and thrombocythemia** 623
Tiziano Barbui and Guido Finazzi

42 **Platelet adhesive protein defect disorders** 639
Kenneth J. Clemetson and Jeannine M. Clemetson

43 **Congenital disorders of platelet secretion** 655
Marco Cattaneo

44 **Congenital platelet signal transduction defects** 674
A. Koneti Rao

45 **Acquired platelet function defects** 689
Joel S. Bennett

46 **Platelet storage and transfusion** 707
Scott Murphy

B **Thrombosis**

47 **Pathophysiology of arterial thrombosis** 727
Lina Badimon, Juan-Jose Badimon and Valentin Fuster

48 **Platelets and atherosclerosis** 738
Peter Verhamme and Paul Holvoet

49 **Platelet involvement in venous thrombosis and pulmonary embolism** 753
Andrew D. Blann and Gregory Y. H. Lip

50 **Gene regulation of platelet function** 760
Thomas J. Kunicki

C **Non-hemostatic disorders**

51 **Platelets and bacterial infections** 783
Mark C. Herzberg and Ke Gong

52 **Interactions of viruses and platelets and the inactivation of viruses in platelet concentrates prepared for transfusion** 807
Laurence Corash

53 **Platelets and parasites** 815
Michel Joseph

54 **Platelets and tumours** 824
Maria Benedetta Donati and Virgilio Evangelista

55 **Platelets and renal diseases** 837
Paola Boccardo, Miriam Galbusera and Giuseppe Remuzzi

56 **Platelets and allergic diseases** 852
Simon Pitchford and Clive P. Page

57 **Platelet interactions with other cells related to inflammatory diseases** 869
Patrice E. Poubelle and Pierre Borgeat

58 **Platelets and the preimplantation stage of embryo development** 885
Christopher O'Neill

59 **Platelets in psychiatric and neurological disorders** 894
B. E. Leonard

60 **Platelets in inflammatory bowel disease** 907
Elizabeth Carty, David S. Rampton and Carole E. Collins

PART IV PHARMACOLOGY

61 **Aspirin** 919
Carlo Patrono

62 **Pharmacology of ticlopidine and clopidogrel** 929
Jean-Pierre Cazenave and Christian Gachet

63 **Platelet membrane glycoprotein (GP) IIb–IIIa antagonists and acute arterial thrombosis** 940
David R. Phillips and Lisa Nannizzi-Alaimo

64 **Other antiplatelet agents** 955
Luigi Iuliano, Fausta Micheletta and Francesco Violi

65 **Pharmacogenetics as a new antiplatelet strategy** 964
Giovanni de Gaetano, Chiara Cerletti and Licia Iacoviello

66 **Cardiovascular gene therapy: implications for platelet vessel wall interactions** 978
Pierre Zoldhelyi, Harold Eichstaedt, Thomas Jax, Janice M. McNatt, Zhi Qiang Chen, Harnath S. Shelat, Harris Rose and James T. Willerson

67 **Pharmacological modulation of the inflammatory actions of platelets** 991
Carla Cicala and Giuseppe Cirino

PART V THERAPY

68 **Design of trials to evaluate antiplatelet agents** 1003
Shamir R. Mehta, John W. Eikelboom and Salim Yusuf

Cambridge Books Online © Cambridge University Press, 2009

69 **Antiplatelet therapy in cardiology** 1013
Herbert D. Aronow and Eric J. Topol

70 **Antiplatelet therapies in neurology** 1040
Graeme J. Hankey

71 **Antiplatelet treatment in peripheral vascular disease** 1055
Raymond Verhaeghe

Afterword 1063
Platelets: a personal story
Gustav V. R. Born

Index 1073

Colour plates between pages 170 and 171

Cambridge Books Online © Cambridge University Press, 2009

Contributors

Charles S. Abrams
University of Pennsylvania
Basic Research Building II/III Room 912
421 Curie Blvd.
Philadelphia
PA 19104, USA

Jan-Willem N. Akkerman
Laboratory for Thrombosis and Haemostasis
Department of Haematology
University Medical Center Utrecht
Heldelberglaan 100
3584 CX Utrecht
The Netherlands

Herbert D. Aronow
Department of Cardiovascular Medicine
The Cleveland Clinic Foundation – Clinic Centre
9500 Euclid Avenue
Cleveland
Ohio, USA

Barrie Ashby
Department of Pharmacology and Sol Sherry Thrombosis
Research Center
Temple University Medical School Philadelphia
PA 19140, USA

Juan-Jose Badimon
Cardiovascular Institute
Mount Sinai School of Medicine
New York, USA

Cambridge Books Online © Cambridge University Press, 2009

Lina Badimon
Cardiovascular Unit
CID/CSIC
Jordi Girona, 18–26
08034 Barcelona, Spain

Tiziano Barbui
Division of Hematology
Ospedali Riuniti
Largo Barozzi 1
24128 Bergamo, Italy

Kurt L. Barkalow
Brigham & Women's Hospital
Hematology Division
LMRC Building, Rm 301
221 Longwood Avenue
Boston
MA 02115, USA

Marc R. Barnard
Center for Platelet Function Studies
University of Massachusetts Medical School
55 Lake Avenue North
Worcester
MA 01655, USA

Joel S. Bennett
University Pennsylvania School of Medicine
Room 914, BRB II/II
421 Curie Blvd.
Philadelphia
PA 19014, USA

Andrew D. Blann
Haemostasis, Thrombosis and Vascular Biology Unit
University Department of Medicine
City Hospital
Birmingham B18 7QH, UK

Paola Boccardo
Mario Negri Institute for Pharmacological Research
Via Gavazzeni 11
24125 Bergamo, Italy

Pierre Borgeat
CRRI
Centre de Recherche du CHUQ and Faculté de Médecine
Université Laval
2705 Boulevard Laurier
Ste-Foy
Qc G1V 4G2, Canada

Gustav V. R. Born
The William Harvey Research Institute
St. Bartholomew's and the Royal London School of Medicine and Dentistry
University of London
Charterhouse Square
London EC1M 6BQ, UK

Lawrence F. Brass
Departments of Medicine and Pharmacology
Center for Experimental Therapeutics
913 BRB II/III
421 Curie Blvd
Philadelphia
PA 19104–6069, USA

Brigitte Brohard-Bohn
U428 INSERM
Faculté de Pharmacie
4 avenue de l'Observatoire
75006 Paris, France

James B. Bussel
Cornell University Medical Center
New York Hospital, USA

Tatiana V. Byzova
Joseph J. Jacobs Center for Thrombosis and Vascular Biology
Department of Molecular Cardiology
Cleveland Clinic Foundation
9500 Euclid Avenue
Cleveland
OH 44195, USA

Elizabeth Carty
Department of Adult and Paediatric Gastroenterology
St. Bartholomew's and The Royal London School of Medicine and Dentistry
London E1 2AD, UK

Marco Cattaneo
Hemophilia and Thrombosis Centre
Department of Internal Medicine
University of Milano
Via Pace 9
20122 Milano, Italy

Cambridge Books Online © Cambridge University Press, 2009

Jean-Pierre Cazenave
INSERM U.311
Etablissement Français du Sang-Alsace
10 rue Spielmann
BP 36
67065 Strasbourg Cedex, France

Chiara Cerletti
Department of Vascular Medicine and Pharmacology
Consorzio Mario Megri Sud
66030 Santa Maria Imbaro, Italy

Zhi Qiang Chen
Wafic Said Molecular Cardiology and Gene Therapy Research Laboratory and Cullen Research Laboratory
Texas Heart Institute
University of Texas Medical School
Houston
Texas, USA

Colin Chesterman
Centre for Thrombosis and Vascular Research
University of New South Wales
Sydney
NSW, Australia

Beng H. Chong
University of New South Wales
WR Pitney Building
St. George Hospital
Belgrave Street
Kogarah, Sidney
NSW 2017, Australia

Carla Cicala
Department of Experimental Pharmacology
University of Naples Federico II
via D. Montesano 49
80131 Naples, Italy

Giuseppe Cirino
Department of Experimental Pharmacology
University of Naples Federico II
via D. Montesano 49
80131 Naples, Italy

Jeannine M. Clemetson
Theodor Kocker Institute
University of Berne
Freiestrasse 1
CH-3012 Berne, Switzerland

Kenneth J. Clemetson
Theodor Kocker Institute
University of Berne
Freiestrasse 1
CH-3012 Berne, Switzerland

Carole E. Collins
West Middlesex University Hospital
Isleworth
Middlesex TW7 6AF, UK

Laurence Corash
Cerus Corporation and Department of Laboratory Medicine
University of California
2411 Stanwell Drive
Concord
San Francisco
CA 94520, USA

Dermot Cox
Centre for Cardiovascular Science
Royal College of Surgeons
Dublin, Ireland

Elisabeth Cramer
INSERM U.474
Hôpital de Port Royal
123 Boulevard de Port-Royal
75014 Paris, France

James L. Daniel
Department of Pharmacology and Sol Sherry Thrombosis Research Center
Temple University Medical School Philadelphia
PA 19140, USA

Rita: De Vos
Morphology and Molecular Pathology
University Hospital Leuven, Belgium

Maria Benedetta Donati
Department of Vascular Medicine and Pharmacology
Istituto di Ricerche Farmacologiche Mario Negri
Consorzio Mario Negri Sud
66030 Santa Maria Imbaro, Italy

Cambridge Books Online © Cambridge University Press, 2009

M. Dreyfus
Unité d'Immunologie Plaquettaire
INTS
6 rue A. Cabanel
75015 Paris, France

Arnaud Drouin
INSERM U.474
Hôpital de Port Royal
123 Boulevard de Port-Royal
75014 Paris, France

Petra Eichler
Institute for Immunology and Transfusion Medicine
Ernst-Moritz-Arndt-University
Greifswald, Germany

Harold Eichstaedt
Wafic Said Molecular Cardiology and Gene Therapy Research Laboratory and Cullen Research Laboratory
Texas Heart Institute
University of Texas Medical School
Houston
Texas, USA

John W. Eikelboom
Department of Haematology
Royal Perth Hospital
Perth, Australia

Virgilio Evangelista
Department of Vascular Medicine and Pharmacology
Istituto di Ricerche Farmacologiche Mario Negri
Consorzio Mario Negri Sud
66030 Santa Maria Imbaro, Italy

Hervé Falet
Brigham & Women's Hospital
Hematology Division
LMRC Building, Rm 301
221 Longwood Avenue
Boston
MA 02115, USA

Richard W. Farndale
University of Cambridge
Department of Biochemistry
Cardiovascular Biology Section
Building O
Downing Site
Cambridge CB2 1QW, UK

Guido Finazzi
Division of Hematology
Ospedali Riuniti
Largo Barozzi 1
24128 Bergamo, Italy

Desmond Fitzgerald
Centre for Cardiovascular Science
Royal College of Surgeons
Dublin, Ireland

Garret A. FitzGerald
Center for Experimental Therapeutics
University of Pennsylvania
421 Curie Boulevard
Philadelphia
PA 19104, USA

A.L. Frelinger III
Center for Platelet Function Studies
University of Massachusetts Medical School
55 Lake Avenue North
Worcester
MA 01655, USA

Kathleen Freson
Centre for Molecular and Vascular Biology
University Hospital Leuven, Belgium

Miha Furlan
Liebeggweg 7
CH-3006 Bern, Switzerland

Mark I. Furman
Center for Platelet Function Studies
University of Massachusetts Medical School
55 Lake Avenue North
Worcester
MA 01655, USA

Valentin Fuster
The Mount Sinai Medical Center
Mount Sinai School of Medicine
Box 1030
One Gustave L. Levy Place
New York
NY 10029, USA

Cambridge Books Online © Cambridge University Press, 2009

Christian Gachet
INSERM U.311
Etablissement Français du Sang-Alsace
10 rue Spielmann
BP 36
67065 Strasbourg Cedex, France

Giovanni de Gaetano
Università Cattolica del Sacro Cuore
Centro di Ricerca e Formazione ad Alta Tecnologia nelle Scienze Biomediche
86100 Campobasso, Italy

Miriam Galbusera
Mario Negri Institute for Pharmacological Research
Via Gavazzeni 11
24125 Bergamo, Italy

Adrian R.L. Gear
Department of Biochemistry and Molecular Genetics
University of Virginia School of Medicine
Charlottesville
VA 22908, USA

Michael H. Gelb
Departments of Chemistry and Biochemistry
University of Washington
Seattle
Washington, USA

Paolo Golino
Division of Cardiology
University of Naples "Federico II"
via Sergio Pansini 5
80131 Naples, Italy

Ke Gong
Department of Preventive Sciences
School of Dentistry
University of Minnesota
17–164 Moos Tower
515 Delaware St.
S.E. Minneapolis
Minnesota 55455, USA

Andreas Greinacher
Institute for Immunology and Transfusion Medicine
Ernst-Moritz-Arndt-University
Greifswald, Germany

Paolo Gresele
Department of Internal Medicine
Section of Internal and Cardiovascular Medicine
University of Perugia
Via E. Dal Pozzo
06126 Perugia, Italy

Philip G. de Groot
University Medical Center
Department of Haematology
Room G03.647
P.O. Box 85500
3508 GA Utrecht
The Netherlands

Thomas A. Haas
Joseph J. Jacobs Center for Thrombosis and Vascular Biology
Department of Molecular Cardiology
Cleveland Clinic Foundation
9500 Euclid Avenue
Cleveland
OH 44195, USA

Graeme J. Hankey
Stroke Unit
Royal Perth Hospital
Wellington Street
Perth
Western Australia 6001

John Hartwig
Brigham & Women's Hospital
Hematology Division
LMRC Building, Rm 301
221 Longwood Avenue
Boston
MA 02115, USA

Ying Hefner
Departments of Chemistry and Biochemistry
University of Washington
Seattle
Washington, USA

H.C. Hemker
Synapse b.v. CARIM
PO Box 616
6200 MD Maastricht
The Netherlands

Cambridge Books Online © Cambridge University Press, 2009

Mark C. Herzberg
Department of Preventive Sciences
School of Dentistry
University of Minnesota
17–164 Moos Tower
515 Delaware St.
S.E. Minneapolis
Minnesota 55455, USA

Paul Holvoet
Cardiovascular Research Unit of the Center for Experimental Surgery and Anesthesiology
KU Leuven
University of Leuven
Campus Gasthuisberg
O&N Herestraat 49
B-3000 Leuven, Belgium

Marc F. Hoylaerts
Center for Molecular and Vascular Biology
KU Leuven MCM groep
Herestraat 49
B-3000 Leuven, Belgium

Licia Iacoviello
Department of Vascular Medicine and Pharmacology
Consorzio Mario Megri Sud
66030 Santa Maria Imbaro, Italy

Luigi Iuliano
University of Rome La Sapienza
Institute of Clinical Medicine 1
00185 Rome, Italy

Thomas Jax
Wafic Said Molecular Cardiology and Gene Therapy Research Laboratory and Cullen Research Laboratory
Texas Heart Institute
University of Texas Medical School
Houston
Texas, USA

Michel Joseph
INSERM IFR 17
Institut Pasteur de Lille, France

Mark Kahn
Department of Medicine
University of Pennsylvania
952 BRB II/III
421 Curie Blvd
Philadelphia
PA 19104–6069, USA

Cécile Kaplan
Unité d'Immunologie Plaquettaire
INTS
6 rue A. Cabanel
75015 Paris, France

Marcelo G. Kazanietz
University of Pennsylvania
Basic Research Building II/III Room 912
421 Curie Blvd.
Philadelphia
PA 19104, USA

John G. Kelton
Department of Medicine
Faculty of Health Sciences
McMaster University
Hamilton
Ontario, Canada

Raelene L. Kinlough-Rathbone
Department of Pathology and Molecular Medicine
McMaster University
Hamilton
Ontario, Canada

E. Anders Kolb
Cornell University Medical Center
New York Hospital, USA

Lori A. Krueger
Center for Platelet Function Studies
University of Massachusetts Medical School
55 Lake Avenue North
Worcester
MA 01655, USA

Thomas J. Kunicki
The Scripps Research Institute
10550 N. Torrey Pines Road
MEM-150
La Jolla
CA 92037, USA

Cambridge Books Online © Cambridge University Press, 2009

Eduardo G. Lapetina
Cato Research Ltd.
200 Westpark Corporate Center
4364 South Alston Avenue
Durham
NC 27713–2280, USA

Brian Leonard
Pharmacology Department
National University of Ireland
Galway, Ireland

Sylviane Levy-Toledano
INSERM U.348
IFR Circulation Lariboisière
Hôpital Lariboisière
Paris, France

Gregory Y. H. Lip
Haemostasis, Thrombosis and Vascular Biology Unit
University Department of Medicine
City Hospital
Birmingham B18 7QH, UK

Francesco Loffredo
Division of Cardiology
University of Naples "Federico II"
via Sergio Pansini 5
80131 Naples, Italy

Roberto Lorenzet
Department of Vascular Medicine and Pharmacology
Consorzio Mario Negri Sud
66030 Santa Maria Imbaro, Italy

Joseph Loscalzo
Whitaker Cardiovascular Institute
Evans Department of Medicine
Boston University School of Medicine
Boston
Massachusetts 02118–2393, USA

Norbet Lubenow
Institute for Immunology and Transfusion Medicine
Ernst-Moritz-Arndt-University
Greifswald, Germany

Janice M. McNatt
University of Texas Medical School
6431 Fannin
MSB 6.043
Houston
TX 77030, USA

John F. Martin
Centre for Cardiovascular Biology and Medicine
British Heart Foundation Laboratories
University College London, UK

Anthony Mathur
Centre for Cardiovascular Biology and Medicine
British Heart Foundation Laboratories
University College London, UK

Shamir R. Mehta
Division of Cardiology
Hamilton Health Sciences Corporation
McMaster University
Hamilton, Canada

Fausta Micheletta
University of Rome La Sapienza
Institute of Clinical Medicine 1
00185 Rome, Italy

Alan D. Michelson
Center for Platelet Function Studies
University of Massachusetts Medical School
55 Lake Avenue North
Worcester
MA 01655, USA

Marina Molino
Istituto di Ricerche Farmacologiche Mario Negri
Consorzio Mario Negri Sud
Santa Maria Imbaro, Italy

Stefania Momi
Department of Internal Medicine
Section of Internal and Cardiovascular Medicine
University of Perugia
Via E. Dal Pozzo
06126 Perugia, Italy

Carine M. Mounier
Département de Biologie
Université de Cergy-Pontoise
95302 Cergy-Pontoise Cedex, France

Cambridge Books Online © Cambridge University Press, 2009

Scott Murphy
American Red Cross Blood Services
Penn-Jersey Region
Philadelphia PA, USA

J. Fraser Mustard
The Founders' Network-CIAR
401 Richmond Street
Suite 281
Toronto
Ontario M5V 3A8, Canada

Lisa Nannizzi-Alaimo
COR Therapeutics, Inc.
256 East Grand Avenue
South San Francisco
CA 94080, USA

Peter J. O'Brien
Genaera Corp.
5110 Campus Drive
Plymouth Meeting, PA 19462, USA

Christopher O'Neill
Human Reproduction Unit
Department of Physiology
University of Sydney
Royal North Shore Hospital
St. Leonards
2065 NSW, Australia

Marian A. Packham
Department of Biochemistry
University of Toronto
Toronto
Ontario M5S 1A8, Canada

Clive P. Page
Department of Pharmacology
King's College London
5th floor
Hodgkin Building
Guy's Campus
London SE1 9RT, UK

Paola Patrignani
Department of Medicine and Aging
Division of Pharmacology
'G. D'Annunzio' University of Chieti
Via dei Vestini, 31
66013 Chieti, Italy

Carlo Patrono
Department of Pharmacology
University of Rome 'La Sapienza'
Rome, Italy

David R. Phillips
COR Therapeutics, Inc.
256 East Grand Avenue
South San Francisco
CA 94080, USA

Simon Pitchford
Sackler Institute of Pulmonary Pharmacology
GKT School of Biomedical Sciences
Guy's Campus
King's College
London SE1 9RT, UK

Edward F. Plow
Joseph J. Jacobs Center for Thrombosis and Vascular Biology
Department of Molecular Cardiology
Cleveland Clinic Foundation
9500 Euclid Avenue
Cleveland
OH 44195, USA

Renata K. Polanowska-Grabowska
Department of Biochemistry and Molecular Genetics
University of Virginia School of Medicine
Charlottesville
VA 22908, USA

Patrice E. Poubelle
CRRI, Room T1–49
2705 Boulevard Laurier
Ste-Foy
Qc G1V 4G2, Canada

V. Proulle
Unité d'Immunologie Plaquettaire
INTS
6 rue A. Cabanel
75015 Paris, France

Fabio M. Pulcinelli
Department of Experimental Medicine and Pathology
University La Sapienze
Rome, Italy

Cambridge Books Online © Cambridge University Press, 2009

David S. Rampton
Department of Adult and Paediatric Gastroenterology
St. Bartholomew's and The Royal London School of Medicine and Dentistry
London E1 2AD, UK

Margaret L. Rand
Division of Hematology/Oncology
The Hospital for Sick Children
Toronto
Ontario, Canada

A. Koneti Rao
Division of Hematology and Thromboembolic Diseases
Temple University Health Sciences Center
Room 300 OMS
3400 N. Broad Street
Philadelphia
PA 19140, USA

Giuseppe Remuzzi
Mario Negri Institute for Pharmacological Research
Via Gavazzeni 11
24125 Bergamo, Italy

Francine Rendu
Centre de médicine préventive cardiovasculaire
Hôpital Broussais
96 rue Didot
75014 Paris, France

Juan A. Rosado
Department of Physiology
University of Cambridge
Downing Street
Cambridge CB2 3EG, UK

Harris Rose
Wafic Said Molecular Cardiology and Gene Therapy Research Laboratory and Cullen Research Laboratory
Texas Heart Institute
University of Texas Medical School
Houston
Texas, USA

Zaverio M. Ruggeri
Division of Experimental Hemostasis and Thrombosis
Department of Molecular and Experimental Medicine
The Scripps Research Institute
10555 North Torrey Pines Road
La Jolla
CA 92037, USA

Stewart O. Sage
Department of Physiology
University of Cambridge
Downing Street
Cambridge CB2 3EG, UK

Maria G. Sciulli
Department of Medicine and Aging
Division of Pharmacology
'G. D'Annunzio' University of Chieti
Via dei Vestini, 31
66013 Chieti, Italy

Sanford J. Shattil
Department of Vascular Biology
The Scripps Research Institute
10550 North Torrey Pines Road, VB-5
La Jolla
CA 92037, USA

Harnath S. Shelat
Wafic Said Molecular Cardiology and Gene Therapy Research Laboratory and Cullen Research Laboratory
Texas Heart Institute
University of Texas Medical School
Houston
Texas, USA

Masamichi Shiraga
Department of Internal Medicine and Molecular Science
Graduate School of Medicine, B5
Osaka University
2–2 Yamada-oka, Suita
Osaka 565–0871, Japan

Pia R-M. Siljander
University of Cambridge
Department of Biochemistry
Cardiovascular Biology Section
Building O
Downing Site
Cambridge CB2 1QW, UK

Jan J. Sixma
University Medical Center
Department of Haematology
Room G03.647
P.O. Box 85500
3508 GA Utrecht
The Netherlands

Cambridge Books Online © Cambridge University Press, 2009

G. Tchernia
Unité d'Immunologie Plaquettaire
INTS
6 rue A. Cabanel
75015 Paris, France

Perumal Thiagarajan
Vascular Biology Research Center and Division of Hematology
University of Texas Medical School
Houston
Texas 77030, USA

Yoshiaki Tomiyama
Department of Internal Medicine and Molecular Science
Graduate School of Medicine, B5
Osaka University
2–2 Yamada-oka, Suita
Osaka 565–0871, Japan

Eric J. Topol
Department of Cardiovascular Medicine
The Cleveland Clinic Foundation – Clinic Centre
9500 Euclid Avenue
Cleveland
Ohio, USA

Mauro Torti
Department of Biochemistry
University of Pavia
via Bassi 21
27100 Pavia, Italy

Chris Van Geet
Department of Paediatrics
University Hospital Leuven, Belgium

Raymond Verhaeghe
Department of Molecular and Cardiovascular Research
University of Leuven Campus
Gasthuisberg O & N
Herestraat 49
Leuven
B-3000, Belgium

Peter Verhamme
Cardiovascular Research Unit of the Center for Experimental Surgery and Anesthesiology
KU Leuven
University of Leuven
Campus Gasthuisberg
O&N Herestraat 49
B-3000 Leuven, Belgium

Jos Vermylen
Centre for Molecular and Vascular Biology
University of Leuven
Herestraat 49
B-3000 Leuven, Belgium

Roberta Vezza
Maxia Pharmaceuticals, Inc.
10835 Altman Row
San Diego, CA 92121, USA

Francesco Violi
University of Rome La Sapienza
Institute of Clinical Medicine 1
00185 Rome, Italy

Jerry Ware
Division of Experimental Hemostasis and Thrombosis
Department of Molecular and Experimental Medicine
The Scripps Research Institute
10555 North Torrey Pines Road
La Jolla
CA 92037, USA

Steve P. Watson
Department of Pharmacology
University of Oxford
Mansfield Road
Oxford OX1 3QT, UK

Kathryn E. Webert
Department of Medicine
Faculty of Health Sciences
McMaster University
Hamilton
Ontario, Canada

Cambridge Books Online © Cambridge University Press, 2009

James G. White
University of Minnesota
Laboratory of Medicine and Pathology
Mayo Box 490
Minneapolis
MN 55455, USA

James T. Willerson
University of Texas Medical School
7000 Fannin, Suite 1700
Houston
TX 77030, USA

Scott Willoughby
Cardiology Unit
The Queen Elizabeth Hospital
28 Woodville Road
Woodville, S. Australia 5011

Kenneth K. Wu
Vascular Biology Research Center and Division of Hematology
University of Texas Medical School
Houston
Texas 77030, USA

Salim Yusuf
Division of Cardiology
Hamilton Health Sciences Corporation
McMaster University
Hamilton, Canada

Pierre Zoldhelyi
Wafic Said Molecular Cardiology and Gene Therapy Research Laboratory and Cullen Research Laboratory
Texas Heart Institute
University of Texas Medical School
Houston
Texas, USA

Cambridge Books Online © Cambridge University Press, 2009

Cambridge Books Online © Cambridge University Press, 2009

Preface

Blood platelets have attracted an increasing interest among clinicians and basic scientists over the last three decades due to the progressive understanding of the role that these cells play in different physiological phenomena and pathological conditions. Indeed, blood platelets not only participate in normal hemostatis and are a central element in atherothrombosis but are also involved in inflammatory and allergic reactions, in some forms of gastroenterological, renal and dermatological disorders, in tumour metastasis and in viral, bacterial and parasitic diseases. It is impressive to realize that blood platelets play a primary role, or are involved, in the diseases responsible for the large majority of disability and death worldwide, and namely not only in myocardial infarction, stroke or peripheral vascular disease but also in cancer and its dissemination and several types of infection. Moreover, platelets, due to their easy sampling and to the fact that they possess a rather complex and complete signal transduction machinery typical of excitable cells, are often taken as a model for neurochemical, biochemical and molecular biology studies. It is also important to underline that, based on the expanding knowledge of platelet biology and pharmacology, recent developments in the field of antiplatelet agents have led to relevant therapeutic advancements with great excitement among cardiologists, neurologists, angiologists and internists.

The wide interest that platelets raise among clinicians and basic investigators is reflected in an explosive, exponential increase of publications involving platelets. It seemed thus useful to finally provide a comprehensive, detailed, up-to-date text for clinicians and basic scientists collecting all the available information on platelet structure, function, participation in disease, pharmacology and therapeutics. Provided the book will meet with the interest of the readership, it is planned that new editions updating the advances on this topic will be published every 3–4 years.

The book has been organized in five sections covering platelet physiology, laboratory methodological aspects, platelet involvement in disease, subdividing the latter into hemostatic and non-hemostatic conditions, platelet pharmacology and finally antiplatelet treatment in the clinic. Within these sections, 72 chapters cover all conventional and unconventional aspects of platelet function, involvement in disease or therapeutics. An emphasis was given to novel or upcoming aspects of platelet physiology, pharmacology and therapeutics, such as gene regulation of platelet function, pharmacogenetics, gene therapy, etc. A large group of leading experts and some of the 'fathers' of modern platelet studies have willingly accepted to contribute to the book, making up an outstanding crew of 157 scientists coming from disparate fields and thus giving a real interdisciplinary view of the subject. All chapters have been edited for homogeneity and to help in providing a balanced view of the various subjects. It is, of course, impossible to avoid some degree of overlap and repetition in such a book, but it was the intention of the Editors that, especially on some important or hot topics, the subject would be discussed and presented from different angles. An effort to provide schemes and tables with easy-to-read take-home messages has been made; extensive and updated bibliographic citations are given to provide the interested reader with access to greater detail than can be included in an already large book. Many investigators and clinicians, even if not primarily interested in platelets, cross the platelet field in their daily work: this book should serve them as a comprehensive and authoritative guide to the diagnosis and therapeutics of diseases involving platelets.

Our thanks go first of all to the authors who have accepted to spend a considerable amount of their busy time in writing their chapters, undergoing pressure to adhere to a tight time schedule and requests to revise and update their work. The preparation of this book would not have been possible without the help of our editorial assistants and of several coworkers in the Institutions of the individual editors. An excellent collaboration with some members of Cambridge University Press, in particular Richard Barling, Mary Sanders and Lucille Murby, has helped in pushing forward what, in some moments, seemed to be a titanic task in a reasonably rapid, though to us exasperatedly slow, way.

Looking back, having forgotten the long tiring hours spent on the project, the preparation of this book has been interesting, enriching and amusing to us; we hope it will also be so for the readers.

The Editors

Paolo Gresele
Clive P. Page
Valentin Fuster
Jos Vermylen

Cambridge Books Online © Cambridge University Press, 2009

Part I

Physiology

Cambridge Books Online © Cambridge University Press, 2009

Cambridge Books Online © Cambridge University Press, 2009

1

History of platelets

J. Fraser Mustard[1,2], Raelene L. Kinlough-Rathbone[1] and Marian A. Packham[3]

[1] *Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada*
[2] *Present address: The Founders' Network-CIAR, 401 Richmond St., Suite 281, Toronto, Ontario, Canada M5V 3A8*
[3] *Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada*

Introduction

In this chapter, we have concentrated on early observations that helped to open up new avenues of research, brought about greater understanding of hemorrhagic and arterial thromboembolic disorders, and in some instances led to new diagnostic methods and novel treatments for the complications of atherosclerosis. Over the years, technological developments have provided more and more tools for investigation. In the 1950s, electron microscopy began to reveal the fine structure of platelets, platelet aggregates, and thrombi. In the 1960s, aggregometers stimulated experiments with aggregating agents and their inhibitors; now, the pace has quickened with the introduction of flow cytometry, molecular biology techniques, monoclonal antibodies, genetically engineered animals and other new approaches.

Findings from research in other fields have frequently been applied to platelets, and knowledge about platelet functions has increased exponentially in the last 50 years. Time and time again, several groups almost simultaneously reported similar new results, but seldom has the rivalry been acrimonious. Some of the key players in the years between 1950 and 1980 have retired or are no longer with us, but this brief history mentions a few of the many contributions they made when the field was young.

Since other chapters will deal with recent findings, we have not attempted to provide a detailed history of the many new topics under investigation, such as the signalling pathways in platelets.

Platelets, hemostasis and thrombosis

In the nineteenth century, only microscopy was available for studies of platelets, which were first detected by Donné in 1842[1]. Although Wharton-Jones in 1852, and Zhan in 1875 described the formation of white thrombi in frogs, they thought that they were made up of leukocytes because thrombocytes in frogs are nucleated[2,3]. In 1882 Bizzozero[4] noted that, in guinea pigs and rabbits, platelets adhered to vascular lesions and to each other, forming a 'white thrombus', and Hayem[5] predicted that a low level of circulating platelets would lead to impaired hemostasis. This suggestion was soon shown to be true[6,7]. In 1885, Lubnitzky[8] pointed out that in flowing blood, platelets have the primary role in the formation of hemostatic plugs that arrest bleeding, and fibrin formation is only secondary. Although the similarities in the composition of hemostatic plugs and arterial thrombi had been recognized by early investigators, these findings were largely ignored until the middle of the twentieth century. They were eventually reemphasized by the Zuckers[9,10] in the late 1940s, and by French, Macfarlane and Sanders[11] in 1964.

Tocantins[12] published a major review of knowledge about platelets in 1938, but platelets were largely disregarded for many years until their contribution to the intrinsic coagulation pathway became of interest. Research during the 1940s and 1950s was focused on blood coagulation because of the need to regulate dosages of dicoumarin drugs[13]; new factors were discovered and eventually the cascade scheme of coagulation was developed by Macfarlane[14]. In it, the role of platelets was recognized as supplying the phospholipid needed for the interaction of factors V and Xa in the intrinsic pathway of coagulation.

However, in the middle of the twentieth century, it was

thought that anticoagulant drugs would inhibit arterial thrombosis as well as venous thrombosis by reducing fibrin formation, because many believed that arterial thrombi were largely blood clots. This idea began to be questioned and interest in platelets increased in 1948 when Duguid[15] revived the concept first developed 100 years before by von Rokitansky[16] that incorporation into the vessel wall of mural thrombi consisting of platelets and fibrin played a role in the development of atherosclerotic lesions. Fisher's observation in 1959 of platelet-rich embolic material passing through retinal vessels during transient monocular blindness[17] further emphasized the importance of platelet thromboemboli in the complications of atherosclerosis. It became clear that studies of the mechanisms involved in platelet adhesion, aggregation, and participation in blood coagulation were relevant to understanding the formation of arterial thrombi and their stability, organization and eventual resolution, as well as the formation of hemostatic plugs and their stability.

Platelets and the endothelium

The conclusion that platelets maintain the integrity of capillary endothelium was derived from perfusion experiments of Danielli[18], and later of Gimbrone and coworkers[19], as well as from several other approaches. In the 1950s, the findings that platelet transfusions controlled the hemorrhagic phase of postirradiation syndromes[20,21] gave credence to Danielli's observation. In other studies by Cronkite and coworkers, autoradiograms of capillaries of X-irradiated rats that had been injected with platelets that were heavily labelled with radioactive sulfur indicated that platelets (or ^{35}S-mucopolysaccharides derived from platelets) were deposited along the capillary walls[22]. Later studies clearly showed that red blood cells and a variety of particles in the circulation crossed capillary walls in thrombocytopenic animals; this did not occur in animals with normal platelet counts[23–25]. The ultrastructural changes of the endothelium that occurred in thrombocytopenia were reversed by platelet transfusions[25,26]. The mechanisms by which platelets support endothelial integrity were explored by Johnson and colleagues[23,27,28], but as Jaffe has pointed out[29], the molecular basis of this important physiological function is still not totally clear.

Platelets and vessel injury

Platelets do not adhere to undamaged endothelium, but when a vessel wall is injured, platelets adhere almost instantaneously – well before fibrin forms[9,10]. In 1959, Bounameaux[30] observed platelet adhesion to subendothelial fibers that were soon shown to be collagen[31]. Zucker and Borrelli[32] reported platelet aggregation in vitro by connective tissue or collagen, and Hovig[33] demonstrated that the aggregating agent ADP was released from platelets stimulated with a saline extract of tendons. Inhibition of collagen-induced aggregation by aspirin was observed in 1967[34–36], and in 1974 and 1975 shown to be due to inhibition by aspirin of the production of prostaglandin endoperoxides and thromboxane A_2 which platelets form when they adhere to collagen[37,38]. It became apparent that ADP and thromboxane A_2 from adherent platelets stimulated platelets flowing by to stick to the adherent platelets and to each other, forming a hemostatic plug or thrombus.

In comparing hemostatic plug formation in normal dogs and dogs with congenital deficiences of coagulation factors, Hovig and colleagues in 1967 observed that the formation of fibrin around the initial mass of aggregated platelets was crucial in stabilizing the plug and preventing rebleeding[39]. This observation indicated the importance of localized thrombin formation around the aggregated platelets at the injury site. Earlier, Jørgensen and Borchgrevink[40] had observed that, in hemophilic subjects, although the time for formation of a hemostatic plug was not appreciably greater than in normal subjects, prolonged rebleeding occurred when the initial hemostatic plug was dislodged, indicating a change in the injured vessel wall (or the platelets adherent to it) that inhibited further platelet accumulation. In accord with this concept, in 1979, Groves and colleagues[41] used ^{51}Cr-labelled platelets to show that following removal of the endothelium from the rabbit aorta, an initial monolayer of platelets formed on the subendothelium, but the number of adherent platelets gradually decreased and the surface attracted fewer and fewer fresh platelets. The reasons for this development of nonthrombogenicity of an injured vessel wall are unknown, but may be relevant to attempts to prevent thrombosis on vascular grafts and in arteries injured by other procedures.

The pattern of blood flow in the arterial circulation determines the events subsequent to platelet adherence at an injury site[42]. In animal experiments in which a vessel wall was injured diffusely, it was shown that only a thin layer of adherent platelets formed when blood flow was laminar, but in regions of disturbed flow, large platelet-rich thrombi formed[43–46].

In 1973, Baumgartner used balloon catheterization to remove the endothelium of rabbit aortas and developed an apparatus in which he could assess the adherence of platelets in flowing, human blood to the subendothelium[47]. In further studies, his group showed platelets adherent to

Cambridge Books Online © Cambridge University Press, 2009

several components of the subendothelium, including collagen[48], and found that platelets from patients with von Willebrand's disease[49] or Bernard–Soulier syndrome[50] adhered poorly.

It was not until the late 1970s that the role of plasma von Willebrand factor in platelet adherence to collagen and the subendothelium under conditions of high shear was recognized[51–54]. (At that time, von Willebrand factor had not been separated from factor VIII, and was often referred to as factor VIII or factor VIII-related antigen.) In accord with these observations about platelet adherence, in 1978 Fuster's group at the Mayo Clinic demonstrated that in pigs with von Willebrand disease platelet adherence to the vascular wall was impaired and the pigs were resistant to the initiation and progression of atherosclerosis[55]. In addition, the pigs had a very severe bleeding tendency.

Fate of arterial thrombi and atherosclerosis

von Rokitansky[16] and Virchow[56] were early investigators who reported that in some instances, the development of atherosclerosis involved vessel wall injury, thrombosis, and the incorportation of thrombi into the vessel wall. In 1887, Welch[57] gave a clear description of arterial thrombi, based on the experiments of a number of investigators, showing that they begin as platelet-rich thrombi and are then transformed into masses rich in fibrin. Much later, these observations were reinforced by Duguid[15], Morgan[58], More and Haust[59] and French[60]. In the 1960s, we observed that platelets were deposited on, and interacted with, the walls of arteries in regions of disturbed blood flow[61–63]; these are sites where atherosclerotic lesions develop and increased vessel permeability is demonstrable.

In 1973, Moore[64] induced 'thromboatherosclerosis' in rabbits by continuous damage of the endothelium of the aorta with an indwelling catheter, and in 1976, he and his group showed that prior administration of anti-platelet serum to induce thrombocytopenia prevented the development of these lesions[65]. The finding by Ross and his colleagues[66] in 1974 that stimulated platelets release a mitogen for smooth muscle cells (platelet-derived growth factor, PDGF) arose from a chance observation. They noticed that serum prepared from platelet-free plasma did not support the proliferation of smooth muscle cells in culture, but when they prepared serum by clotting platelet-rich plasma, the serum supported cell growth as effectively as serum prepared from whole blood. These results gave even more credence to the theory that platelets are involved in the development of atherosclerotic plaques because they promote the proliferation of smooth muscle cells.

The progression of atherosclerotic plaques also involves platelets since platelet-rich thrombi have been shown to be incorporated into the vessel wall at sites of injury[15,59,67]. Platelet-rich thrombi that form on ruptured atherosclerotic plaques may occlude the lumen of a vessel, or may embolize. When the thromboemoli impact in smaller, downstream vessels, organ damage occurs[68–70].

These concepts about platelets and atherosclerosis have stood the test of time. The picture developed by Ross[71] of the development of an atherosclerotic plaque is now well known, and the signalling pathways PDGF activates are being explored[72]. The importance of platelet interaction with the components of ruptured atherosclerotic plaques in the thromboembolic complications of atherosclerosis is now generally accepted[73].

Clot retraction

One of the earliest recorded observations, eventually shown to be a function of platelets, was clot retraction, described by Hewson in the late eighteenth century[74]. About 100 years later, in 1878, Hayem reported that platelets had a tendency to change their form, aggregate, and interact with fibrin strands[75]. Although he called platelets 'hématoblastes' because he thought they were precursors of erythrocytes, he counted them accurately and described their role in clotting and in clot retraction. In 1906, Le Sourd and Pagniez[76] established the unique ability of platelets to cause clot retraction. This early history was reviewed by Budtz-Olsen[77] in 1951, in connection with his studies of clot retraction, and in the same year, Øllgaard[78] described using clot retraction as a quantitative test of the function and agglutinability of platelets. Bettex-Galland and Lüscher[79] later showed that metabolic energy was required for clot retraction.

Source of platelets

For many years after the discovery of platelets there was considerable disagreement about their source. Wright's work in the early 1900s with stained sections of tissue and supravital preparations, provided the first evidence that platelets were derived from megakaryocytes[80,81], but it was the work of Humphrey[82] in 1955, on the basis of studies with fluorescent antibodies, that provided irrefutable evidence that the megakaryocyte was the precursor cell of platelets. Finally, Kinoshita and colleagues[83] in 1961 developed a technique for placing windows in bone that allowed the marrow to grow in the thin space between the windows

Cambridge Books Online © Cambridge University Press, 2009

where platelet production from megakaryocytes could be observed directly.

Thrombopoietin

Although the existence of a substance responsible for controlling platelet production (thrombopoietin, TPO) had been postulated for many years, all attempts to isolate it had failed. In 1992, the proto-oncogene c-mpl was cloned, leading to the identification of TPO. In 1994, five groups were able to either purify TPO or clone the cDNA. Since then, there has been an impressive amount of research on this topic, reviewed by Kaushansky[84] in 1999.

Platelet lifespan

The initial approaches used to determine platelet lifespan fall into three main categories: (i) Depletion studies, in which thrombocytopenia was induced and the rate of platelet regeneration determined[85]; (ii) Transfusion studies in which normal platelets were transfused into thrombocytopenic individuals and the platelet count monitored[86,87] (this gave considerable variability in assessments of lifespan depending on the nature and extent of the thrombocytopenia); and (iii) Labelling techniques in which various radioisotopes were introduced into platelets and the length of time that radioactivity remained in the circulation was measured. More recently, particularly in infants, a return of the ability of platelets treated with aspirin to produce malondialdehyde was used to measure platelet survival[88]. The most commonly used method now involves labelling with ^{51}Chromium[89] or ^{111}Indium[90].

It is now generally accepted that the normal lifespan of platelets in humans is 9–11 days, but it is not clear whether this is a measure of the role of platelets in maintaining the integrity of the vascular tree. Thrombin-induced release of granule contents in vivo or in vitro does not shorten platelet survival[91,92], but when platelet membrane glycoproteins have been cleaved by proteolytic enzymes the platelets do not survive in the circulation[93]. Platelet survival tends to be shortened in individuals with diseases associated with thrombosis and thromboembolism[94,95].

The controversy over the question of how platelet survival curves should be analysed (linear or exponential) was settled in 1971 when Murphy and Francis introduced the gamma function method of calculation and the International Committee for Standardization adopted it[96,97].

Platelet transfusion

The first transfusion in man is said to have been carried out in 1667 by Jean Baptiste Dennis who transfused a young man with the blood of a lamb[98]. Italian physicians in the early part of the twentieth century are known to have employed blood transfusions for the treatment of thrombocytopenic purpura, but since anticoagulants were not available at that time, transfusion was performed with paraffin-coated syringes. Gradually, there were improvements in transfusion techniques to avoid activation of platelets. These included the introduction of silicones to provide non-wettable surfaces for glass containers, differential centrifugation techniques under temperature control for the separation of blood components, the introduction of plastic containers, and refinement of the composition of anticoagulant solutions[99]. The conditions of storage have been readjusted frequently to provide incremental improvements in the viability and function of platelet concentrates for transfusion[100].

Isolation of platelets

In the early 1970s, we developed methods to isolate platelets, wash them, and resuspend them in artificial media so that they retain most of the properties of platelets in plasma[101,102]. Important conditions were determined to be the choice of anticoagulant, use of a balanced salt solution (Tyrode's) containing a physiological concentration of calcium, a source of metabolic energy (glucose), inclusion of a system (e.g. apyrase) to remove ADP that leaks from the platelets, and the addition of a protective protein (albumin)[103].

Platelets have also been separated from plasma on an albumin gradient[104] or by gel filtration[105–107].

Since the artificial media in which platelets are resuspended can be readily modified, and the formation of fibrin does not interfere with studies involving thrombin, these isolation techniques have allowed considerable advances in the elucidation of the mechanisms underlying platelet functions.

Platelet aggregation and platelet receptors

Up until 1963, platelet aggregation was observed by visual or microscopic examination of platelet-rich plasma when it was shaken with an aggregating agent. In 1962 Born[108] published his paper describing the development of the platelet aggregometer and shortly thereafter O'Brien[109]

Cambridge Books Online © Cambridge University Press, 2009

Table 1.1. Highlights of platelet research

Year	Event
1842	Platelets detected microscopically
1882	Platelet adherence to damaged blood vessels and aggregation
1882	Thrombocytopenia shown to impair hemostasis
1885	Platelets shown to have primary role in formation of hemostatic plugs
1906	Platelets shown to be derived from megakaryocytes
1906	Platelets shown to cause clot retraction
1918	Glanzmann's thrombasthenia recognized
1938	Major review by Tocantins
1948	Incorporation of thrombi into vessel wall shown to be involved in the development of atherosclerotic lesions
1948	Bernard-Soulier syndrome described
1950s	Electron microscopy began to reveal the fine structure of platelets
1950s	Serotonin recognized as a platelet constituent taken up from plasma
1950s	Platelets shown to maintain integrity of capillary endothelium
1959	Platelets shown to contain a contractile protein – thrombosthenin
1959	Transient monocular blindness due to platelet emboli
1959	Platelets shown to adhere to collagen
1961	ADP from red cells identified as an aggregating agent
1962	Aggregometer based on light transmission developed
1963	Collagen and thrombin shown to release ADP from platelets
1963	Epinephrine shown to aggregate platelets
1964	Introduction of use of ^{14}C-serotonin to measure release of granule contents
1967	Aspirin shown to inhibit collagen-induced aggregation and release
1967	Stimulated platelets shown to give rise to microparticles
1967	Prostaglandin E_1 shown to inhibit aggregation
1970	Storage pool disease recognized
1971	Agglutination of platelets by ristocetin developed as a means of detecting vWD and later, Bernard-Soulier syndrome
1971	Aspirin shown to inhibit formation of prostaglandins in platelets
1972	Platelet-activating factor (PAF) recognized
1972	Methods developed for isolation and resuspension of platelets in artificial media
1973	Arachidonic acid shown to aggregate platelets

Year	Event
1974	Role of phosphoinositides in platelet functions recognized
1974	Release of platelet-derived growth factor (PDGF) by stimulated platelets
1974	Abnormal glycoprotein pattern noted in thrombasthenia platelets
1975	Thromboxane A_2 found to be an aggregating agent whose formation is inhibited by aspirin
1975	Ticlopidine developed as an inhibitor of ADP-induced aggregation
1976	Prostacyclin (PGI_2) identified as a strong inhibitor of platelet reactions
1970s	Plasma von Willebrand factor implicated in platelet adherence to collagen and the subendothelium under conditions of high shear
1978	Fibrinogen shown to bind to platelets during aggregation
1979	Identification of a patient with a defect of platelet procoagulant activity (Scott syndrome)
1980	Investigations of receptors, G-proteins, signalling
1981	Fibrinogen receptor identified as glycoprotein IIb/IIIa (αIIbβ3)
1982	Fibrinogen γ-chain identified as a binding site for platelets
1982	Fluorescent indicators used for measurement of cytosolic calcium
1983	Monoclonal antibodies to GPIIb/IIIa developed
1984	P-selectin identified as an α-granule constituent that can be detected by flow cytometry on the platelet surface after stimulation
1985	Peptides with RGD sequence shown to inhibit binding of fibrinogen and vWf to platelets
1986	Defects in collagen receptors on platelets identified
1988	Meta-analysis of 25 clinical trials of non-steroidal anti-inflammatory drugs show 25% reduction in risk of a thromboembolic event
1991	Thrombin shown to activate platelets by cleavage of a protease-activated receptor
1992	Identification of a patient with platelets unresponsive to ADP
1994	Thrombopoietin purified
1990s	Many inhibitors of fibrinogen binding to platelets entered clinical trials
1990s	Three ADP receptors identified

Cambridge Books Online © Cambridge University Press, 2009

described a similar device. As a result, it became possible to assess the rapidity and extent of aggregation by recording changes in light transmission through a stirred suspension of platelets. Almost immediately, other investigators adopted this technique and during almost 40 years it has had the major role in tens of thousands of studies of aggregating agents, inhibitors of aggregation, and conditions that influence aggregation.

Thrombin

Eberth and Schimmelbusch[110] in 1888 and Wright and Minot[111] in 1917 used the term 'viscous metamorphosis' to describe the changes in platelets when they stick to surfaces and to each other. Because thrombin induced these changes, it was originally thought that fibrin formation was involved in platelet-to-platelet adherence[112]. It was not until 1946 that this concept was questioned by Pinniger and Prunty[113] who showed that thrombin aggregated platelets in the blood or platelet-rich plasma of patients with afibrinogenemia. Following the demonstration by Gaarder and her colleagues[114] that ADP from red cells could cause platelet aggregation and Grette's observation that thrombin induces the release of ADP from platelets[115], Haslam suggested that released ADP was responsible for thrombin-induced aggregation[116]. This conclusion was based on his observation that enzymatic removal of ADP was inhibitory. However, other investigators, including ourselves[117], showed that thrombin could aggregate platelets even when released ADP was removed and thromboxane A_2 formation was blocked with aspirin or indomethacin. This observation led to the concept of a 'third pathway' responsible for thrombin-induced aggregation. At one time, formation of platelet-activating factor (PAF) by thrombin-stimulated platelets was considered as a candidate for this role[118], but further studies have not supported this suggestion[119,120].

It was not until 1991 that the way in which thrombin stimulates platelets was elucidated by Coughlin's group[121]. They obtained evidence for a unique protease-activated receptor, now designated PAR-1. The finding that platelets from some species did not respond to the newly exposed peptide, SFLLRN, whereas human platelets did so[122,123], indicated that there might be other receptors for thrombin, and Coughlins's group has now identified PAR-3 on mouse platelets and PAR-4 on mouse and human platelets. The development of knowledge about these receptors has been reviewed recently by Coughlin[124].

Collagen

Interest in collagen as an aggregating agent arose from observations in the early 1960s that, when blood vessels were injured, platelets adhered to collagen[30,31] and in doing so, released ADP[32,33]. Aspirin inhibited collagen-induced aggregation[34–36], an effect shown to be caused by inhibition of cyclo-oxygenase[125]. It became apparent that platelets adherent to collagen were stimulated to release ADP and form thromboxane A_2, and that these aggregating agents acted synergistically to cause aggregation of the non-adherent platelets[117].

The nature of the collagen receptor on platelets has been controversial with GPIa/IIa, GPVI and GPIV as candidates. Hemostasis is impaired in patients with a deficiency of GPIa/IIa[126] or GPVI[127], but a defect in GPIV does not lead to hematologic problems[128]. The current concept is that both GPIa/IIa and GPVI are involved in platelet adherence to collagen[129,130]. (See Chapter 11.)

ADP

The finding by Hellem[131] and by Øllgaard[132] that a factor from red blood cells caused platelet aggregation was quickly followed in 1961 by its identification as ADP by Gaarder and her coworkers[114] in Owren's Institute for Thrombosis Research in Oslo. At first ADP was considered to be the factor responsible for aggregation by all the agonists that caused its release from platelets[116]. Later, it became apparent that this concept was untenable, although the role of released ADP in potentiating aggregation by collagen, thrombin and other release-inducing agents is well accepted.

In some species (including man), ADP-induced aggregation was observed to occur in two phases in citrated platelet-rich plasma[133–135], the second phase being associated with the release of serotonin and ADP[133–137], and not readily reversible. (Subsequent studies showed that thromboxane A_2 formed under these conditions was responsible for secondary aggregation[138].) However, there were several observations showing that ADP-induced aggregation in vivo was reversible[69,133,139], leading to the suggestion by Mustard and Packham[140] in 1970 that 'this apparent ability of ADP to induce irreversible platelet aggregation in vitro may be an artifact of the experiments'. In 1975 we reported that the low calcium concentration produced by the use of citrate as the anticoagulant was responsible for the second phase of ADP-induced aggregation[141]; it does not occur in platelet-rich plasma from blood anticoagulated with heparin, hirudin or PPACK[142,143]. Nevertheless, because so

Cambridge Books Online © Cambridge University Press, 2009

many studies of platelet aggregation are done in citrated platelet-rich plasma, the notion has persisted that ADP causes extensive release of granule contents and the formation of thromboxane A_2. Failure to understand this artefact has sometimes led to misleading conclusions.

In the early 1970s, two groups[144,145] reported that ADP inhibits the increase in cyclic AMP caused by exposure of platelets to prostaglandin E_1; the ADP receptor responsible for this down-regulation of adenylyl cyclase has been identified recently[146]. It is now known that metabolites of the thienopyridine derivative, ticlopidine, and its analogue, clopidogrel, which are specific inhibitors of primary ADP-induced aggregation[147–149], irreversibly block this ADP receptor[150–152]. The unstable, active metabolite of clopidogrel has recently been identified[153]. Clinical trials of these drugs have been carried out[154–156] (See Chapters 9 and 62).

Following the discovery of individuals whose platelets do not aggregate in response to ADP[157], interest intensified in ADP receptors, three of which have been identified (Chapter 9).

Arachidonic acid and thromboxane A_2 (TXA_2)

Aggregation by arachidonic acid was reported in 1973 by Silver and colleagues[158] and shown to be due to its conversion to PGG_2, PGH_2 and TXA_2[38,125]. Receptors for these products have been characterized (Section 1, Chapter 9) and some abnormalities have been identified.

Serotonin

The flurry of interest in serotonin began in the 1950s when Rand and Reid[159] discovered that platelets contained it. Serotonin was shown to be released from platelets by Bigelow[160] and by Zucker and Borrelli[161] and actively taken up by them[162,163] into the dense granules[164,165]. Platelets carry all the serotonin in blood. Serotonin was demonstrated to be a weak aggregating agent by Mitchell and Sharp[166] in 1964, and to act synergistically with other agonists by Baumgartner and Born[167] in 1968. The extensive research on serotonin up to 1970 was summarized by Mustard and Packham[140]. At that time, platelets were beginning to be recognized as a model for serotonergic neurons[168].

Epinephrine (adrenaline)

In the early 1960s, O'Brien[169] and Mitchell and Sharp[166] discovered that epinephrine and norepinephrine caused platelets to aggregate in two phases in citrated human platelet-rich plasma. Haslam[170] examined the role of ADP in epinephrine-induced aggregation and concluded that ADP was involved in both phases.

The ability of epinephrine to potentiate aggregation by other agonists was first noted by Ardlie and colleagues[171] in 1966 and further documented in 1967 by Mills and Roberts[172]. It is now apparent that epinephrine by itself is not an aggregating agent, but acts in a strong, synergistic fashion with other aggregating agents[173,174].

Epinephrine acts by stimulation of alpha adrenergic receptors[172,175] and causes a decrease in cyclic AMP in platelets if its concentration has been raised by prostaglandin E_1[176].

Platelet activating factor (PAF)

In 1972 Benveniste, Henson and Cochrane[177] introduced the term 'platelet-activating factor' for the soluble factor, released from basophils upon IgE stimulation, that caused aggregation and release by rabbit platelets. Seven years later, Hanahan's group identified PAF as a phosphoacyl glycerol[178]. Although human platelets are aggregated by PAF, the concentration required is much higher than for aggregation of rabbit platelets[119,179]. PAF is synthesized by rabbit platelets when they are stimulated by thrombin, but it does not appear to play a part in thrombin-induced aggregation of human platelets[119,120]. The PAF receptor has been investigated by Izumi and Shimizu[180].

Ristocetin, von Willebrand factor, and glycoprotein I

Ristocetin was withdrawn from clinical use as an antibiotic because many patients who received it became thrombocytopenic. In 1960, Gangarosa and colleagues[181] noted aggregation of platelets in vitro upon the addition of ristocetin, but it was not until 1971 that Howard and Firkin[182] confirmed this observation and introduced ristocetin as 'a new tool in the investigation of platelet aggregation'. They showed that ristocetin could be valuable in subdividing types of von Willebrand disease. Platelets fixed with paraformaldehyde agglutinate in response to ristocetin in normal plasma (but not in plasma from patients with some types of von Willebrand's disease) and this reaction has become a standard clinical test for von Willebrand's disease[183].

In 1973, two groups reported that platelets from patients with Bernard–Soulier syndrome (BSS)[184] did not aggregate

Cambridge Books Online © Cambridge University Press, 2009

in response to ristocetin[185,186]. Weiss and coworkers[50] suggested that BSS platelets lacked a receptor for von Willebrand factor involved in platelet adherence, and in 1975, Nurden and Caen[187] reported that a major glycoprotein (GPI) was not visible among the membrane proteins of platelets from patients with BSS. The current state of knowledge about BSS has been reviewed recently by López and colleagues[188].

Fibrinogen, von Willebrand factor, and GPIIb/IIIa

Although it was known as early as 1964 that fibrinogen was required for ADP-induced aggregation of human platelets[189,190], it was not until 1978 that we reported the rapid association and dissociation of radiolabelled fibrinogen during ADP-induced platelet aggregation and deaggregation[191]. The concept was developed that ADP induces a saturable receptor for fibrinogen on human platelets[192–194] which is missing on the platelets of patients with Glanzmann's thrombasthenia[195]. Nurden and Caen[196] had already noted an abnormal glycoprotein pattern in the membranes of platelets from patients with this disorder. In 1981, the fibrinogen receptor was identified as GPIIb and IIIa forming a calcium dependent complex on the surface of activated platelets[197,198]. Meanwhile, Pierschbacher and Ruoslahti[199] had identified Arg–Gly–Asp (RGD) as the amino acid sequence in the cell binding domain of fibronectin responsible for its attachment to fibroblasts. This RGD sequence also occurs in both fibrinogen[200] and von Willebrand factor[201] and peptides containing this sequence were shown by several groups to inhibit the binding of these proteins to activated platelets[202–204]. A different binding site for GPIIb/IIIa on fibrinogen had been found at the C-terminus of the gamma chain, which includes the dodecapeptide sequence 400–411[205], and it now appears that it is more important for the binding of fibrinogen to human platelets than is the RGD sequence, as shown by Farrell and colleagues[206,207] using mutant fibrinogen molecules. The result of the finding about the inhibitory effect of RGD peptides has been the development of drugs containing this sequence as inhibitors of platelet aggregation in patients during percutaneous coronary intervention or as treatment of acute coronary syndromes[208].

Another approach to inhibition of platelet aggregation began in 1983 from the use by Coller and his colleagues[209] of hybridoma technology to produce monoclonal antibodies to GPIIb/IIIa (10E5 and 7E3). In 1995, Coller[210] reviewed the development of a mouse/human chimeric monoclonal antibody fragment (c7E3 Fab, abciximab, ReoPro™) for use in patients to reduce complications after angioplasty. Clinical trials of its efficacy have been carried out[208].

Platelets in infectious diseases and immune reactions

For many years there has been considerable interest in platelet interactions with tumor cells, antigen–antibody complexes, viruses, bacteria and bacterial products such as endotoxin. These subjects have been reviewed elsewhere[94,211], and are discussed in Section III.C.

Platelet granules and the release reaction

Although it is now known that platelets have three main types of storage granules – dense or amine storage granules containing adenine nucleotides, serotonin and divalent cations; alpha granules containing many proteins; and lysosomal granules containing acid hydrolases – in the early days these distinctions had not been made.

In the 1950s, serotonin was the first substance to be recognized as originating from platelets during blood coagulation[160,212]. Then Humphrey and Jaques[213] noted the release of histamine and serotonin from platelets by antigen-antibody reactions. Grette[115] observed thrombin-induced release of serotonin, adenine nucleotides and fibrinogen in 1962 and introduced the term 'release reaction'. Zucker and Borrelli[32] and Hovig[33] reported collagen-induced release of ADP, and in 1964, Spaet and Zucker[214] showed that collagen also released ^{14}C-serotonin previously taken up in vitro. This method of measuring the extent of the release of dense granule contents has been widely used ever since.

Haslam[116,170] was the first to draw attention to the role of the release reaction; he showed that when an extracellular ADP-removing system was present, aggregation by several agents was inhibited, thus obtaining evidence that released ADP was responsible for aggregation. Haslam also proposed that ADP was released directly and was not the product of hydrolysis of ATP. In 1964, Buckingham and Maynert[215] suggested that the substances released had different subcellular localizations from those retained, and in keeping with this suggestion, in 1967 Ireland[216] showed that thrombin induced the release of metabolically inactive nucleotides while the active ones were retained by the platelets. Mills and his colleagues[137] showed that ADP was released during the second phase of ADP-induced aggregation. Three groups reported that strong aggregating

Cambridge Books Online © Cambridge University Press, 2009

agents (but not ADP or epinephrine) caused the release of lysosomal enzymes[137,217,218]. In 1969, release of platelet factor 4 and calcium were reported[219,220], and a major review about the release reaction was written by Holmsen, Day and Stormorken[221]; they pointed out that the process consists of extrusion of material from the platelet granules to the external medium in a process similar to secretion. At that time it was known that the metabolically inert adenine nucleotides were located in the dense granules, but lysosomal enzymes were thought to be in α-granules that had been differentiated from dense granules by electron microscopy. In 1977, Fukami and Salganicoff[222] reviewed the evidence for the various types of storage organelles in platelets, pointing out that in addition to dense granules and alpha granules, there may be at least two types of lysosomal granules.

A hereditary defect caused by a deficiency in the storage pool of adenine nucleotides (storage pool disease) was discovered by Holmsen and Weiss[223] in 1970. Subsequently, a number of congenital abnormalities of granule contents or their release have been identified.

Mustard and coworkers[224] reported release of a permeability factor in 1965, and in 1974 Ross and his colleagues[66] discovered that a platelet-derived growth factor (PDGF) for smooth muscle cells was released from blood platelets upon clotting of blood or platelet-rich plasma.

The finding in the mid-1980s that an alpha granule membrane protein (PADGEM, GMP 140, P-selectin, CD62P) appeared on the surface of platelets as their contents were released[225–227] has led to its detection by flow cytometry as a means of determining the extent of release of alpha granule contents[228]. P-selectin has been shown to mediate adhesion of platelets to neutrophils and monocytes[229].

Platelet procoagulant activity, PF3, and microparticles

Activated platelets contribute to coagulation by providing a catalytic phospholipid surface for the 'tenase' and 'prothrombinase' reactions. As early as 1936, Chargaff and coworkers[230] demonstrated that a phospholipid fraction from horse platelets accelerated clotting in cell-free chicken plasma, but this finding was ignored, possibly because of the cross-species complication. It was not until the early 1950s that van Crevald and Paulssen[231,232] and Alkjaersig, Abe and Seegers[233] studied the role of platelets in coagulation and called the procoagulant material platelet factor 3 (PF3, now known as platelet procoagulant activity or PCA). For some time there was debate about how this activity became available on the surface of platelets since it appeared to be masked on intact platelets[234]. In 1965, Hardisty and Hutton[235] concluded that adhesion of platelets to an activating surface was necessary for an optimal effect, and that ADP-induced aggregation alone did not make PF3 available. In the same year, Spaet and Cintron[236] showed that connective tissue particles caused platelets to develop PF3 activity. Later, Joist and colleagues[237] used washed platelets in an artificial medium to demonstrate that PF3 becomes available on the surface of platelets that have undergone a release reaction.

In the 1980s, phosphatidyl serine (PS) was identified as responsible for the procoagulant activity of stimulated platelets[238,239], and the evidence obtained from experiments with red cells was applied to platelets regarding the asymmetric nature of membrane phospholipid distribution. Between 1987 and 1996, three enzymes – an ATP-dependent translocase, a floppase, and a scramblase – were shown to be responsible for transbilayer movement of phospholipids and the exposure of PS on the surface of stimulated platelets[240–243]. The identification in 1979 by Weiss and coworkers of a patient with a defect in platelet procoagulant activity (Scott syndrome) has contributed significantly to our understanding of these mechanisms[244,245].

A characteristic event that accompanies the availability of platelet procoagulant activity is the shedding of membrane-derived microvesicles or microparticles from the surface of activated platelets. Although these microparticles were reported by Wolf[246] in 1967, only a few investigators took an interest in them[247–249] until Sims and his group[250] in 1988 began investigating the effect on platelets of the membrane attack complex of the complement system (C5b-9) and found that membrane vesicles were released. These microparticles provided a catalytic surface for the prothrombinase complex and were highly enriched in alpha-granule-derived factor V (or Va); they also contained GPIb, GPIIb/IIIa and P-selectin. Microparticles are also formed when platelets are stimulated with the calcium ionophore A23187, or with the combination of thrombin and collagen[251], and they have been detected in the blood of patients exhibiting intravascular platelet destruction[252]. Flow cytometry has facilitated the study of microparticles[228]. It has been speculated that since microparticles possess many of the properties of intact platelets, they might be stored and transfused as agents promoting hemostasis[253].

Cambridge Books Online © Cambridge University Press, 2009

Aspirin

Although prolongation of the bleeding time by aspirin had been recognized as early as 1955 by Beaumont and coworkers[254], and then by others[255,256], including Quick in 1966[257], it was not until 1967 that several groups of investigators reported that aspirin inhibited platelet aggregation and the release of granule constituents.

Our group's involvement in the discovery of the inhibitory effect of aspirin on platelet aggregation began as the result of serendipity. In 1963, we were measuring platelet survival times in elderly, atherosclerotic World War I veterans, many of them suffering from gout[258]. Upon examining the data, we realized that those who were taking the uricosuric agent sulfinpyrazone had longer platelet survival times than those who were not receiving this medication. We then showed that administration of sulfinpyrazone prolonged shortened platelet survival and reduced platelet adhesiveness to glass[259]. This finding led to in vitro tests of the effect of the pyrazole compounds, sulfinpyrazone and phenylbutazone, on platelet aggregation induced by several agonists, and the discovery that these non-steroidal anti-inflammatory drugs blocked aggregation induced by collagen, antigen–antibody complexes and gamma globulin-coated surfaces and inhibited release of adenine nucleotides, although the drugs did not affect the primary phase of ADP-induced aggregation[260]. In addition, with rabbits, intravenous infusion of sulfinpyrazone or phenylbutazone impaired hemostatic plug formation at the ends of transected mesenteric vessels[260,261]. Since aspirin was a well-known anti-inflammatory drug, we tested it in vitro and reported similar inhibitory effects in June of 1967 at the Oak Ridge Platelet Symposium[34]. (A full paper about these findings was published in 1968[35].) At this time, Weiss and Aledort[36] were following up on Quick's[257] observation of prolonged bleeding times in subjects who had taken aspirin, and observed that aspirin ingestion impaired collagen-induced aggregation and inhibited release of ADP. Also in 1967, Zucker and Peterson[262] noticed that the second phase of ADP-induced aggregation did not occur in citrated platelet-rich plasma from individuals who had consumed aspirin, and O'Brien[263] found that epinephrine-induced aggregation in citrated platelet-rich plasma was also inhibited; this inhibitory effect persisted for 2–6 days after ingestion of a single subclinical dose. Although it was known from experiments with ^{14}C-labelled acetylsalicylic acid in 1969 that a protein in platelets was acetylated by aspirin[264], it was not until 1975 that Roth and his colleagues[265] demonstrated irreversible acetylation of cyclooxygenase by aspirin, thus accounting for the persistence of the aspirin effect.

In the early 1990s, it was recognized that there are two isoforms of cyclooxygenase, a constitutive form, COX-1, and an inducible form, COX-2. Only COX-1 is present in platelets, whereas COX-2 is expressed in other cells after an inflammatory insult. Aspirin and other non-steroidal anti-inflammatory drugs inhibit both COX-1 and COX-2, but drugs have now been developed that selectively inhibit COX-2, for the treatment of arthritis and possibly other conditions[266].

With the realization that platelet aggregation could be inhibited by sulfinpyrazone or aspirin, large scale clinical trials were launched in the early 1970s, before there was a thorough understanding of the enzymatic reactions in platelets that were inhibited by these drugs. The earliest trials varied in their designs, but they all involved male subjects who had already suffered from myocardial infarction, and the end points were short term reinfarction or death[267]. Later trials of secondary prevention enrolled patients who had a history of stroke, transient cerebral ischemia, myocardial infarction, or unstable angina. By the late 1980s, it was calculated that meta-analysis of 25 trials showed that aspirin caused a 25% reduction in the risk of developing a subsequent important vascular event[268,269]. Interestingly, these clinical trials were well under way before it was discovered that in the 1950s, Craven[270,271] had reported in the *Mississippi Valley Medical Journal* that aspirin prevented coronary and cerebral thrombosis. He had studied approximately 8000 men taking 5 to 10 grains of aspirin daily.

Aspirin is now routinely administered to patients at risk of the thromboembolic complications of coronary artery disease. The demonstration by Barnett and colleagues that aspirin reduced the incidence of transient ischemic attacks and ischemic stroke[272] has led to its continued use in this condition also[273]. However, there has been controversy over the most suitable dose of aspirin, with suggestions of as little as 40 mg every other day as an appropriate dose.

Prostaglandins

While the findings that sulfinpyrazone and aspirin inhibited platelet aggregation were unfolding, research on prostaglandins was accelerating although, initially, it did not involve platelets. Prostaglandins had been isolated from human seminal plasma and sheep seminal vesicles in the early 1930s and crystallized in 1957[274]. The prostaglandins are formed from polyunsaturated fatty acids. It was not until 1967 that there were reports of PGE_1 inhibiting platelet aggregation[275–277]; it was shown to increase cyclic AMP in platelets by stimulation of adenylyl cyclase[278]. Zucker[279]

Cambridge Books Online © Cambridge University Press, 2009

had reported in 1965 that cyclic AMP inhibited platelet aggregation and the release reaction.

However, PGE_1 is not produced by platelets; it was PGE_2 and $PGF_{2\alpha}$ that were first detected as being formed by platelets during aggregation, and neither of them caused platelet aggregation[280]. In 1969, Piper and Vane[281] reported that formation of an unstable 'rabbit aorta contracting substance' (RCS) from sensitized guinea pig lungs was inhibited by aspirin-like drugs; the activity of this short-lived substance was later shown to be due to prostaglandin endoperoxides[282], designated PGG_2 and PGH_2[125]. Two publications showing that aspirin inhibited the formation of prostaglandins were published simultaneously in 1971[283,284], although only one of them dealt with platelets[283]. In 1973, arachidonic acid was reported to aggregate platelets[158], and platelets aggregated by arachidonic acid were shown to release RCS[285]. In 1974, three groups showed that the endoperoxides PGG_2 and PGH_2 were formed by platelets and released into the medium upon stimulation by collagen, epinephrine, arachidonic acid, or thrombin[37,125,286,287]. Also in 1974, Hamberg and his colleagues isolated and determined the structure of PGG_2 and PGH_2 that cause platelet aggregation and proposed that aspirin inhibited the cyclooxygenase responsible for their formation[125]. In 1975, Hamberg and his coworkers identified thromboxane A_2 as a short-lived, potent inducer of platelet aggregation that was derived from the prostaglandin endoperoxides[38]. It is now well established that the inhibitory effects of aspirin on platelet functions are due to its irreversible acetylation of cyclooxygenase, thus preventing the formation of the prostaglandin endoperoxides and thromboxane A_2[288].

In 1976, Moncada and his colleagues in Vane's group[289] discovered PGX (later named PGI_2 or prostacyclin) as a prostaglandin metabolite formed from arachidonic acid in vascular tissue; prostacyclin is a strong inhibitor of platelet aggregation and thrombus formation[290]. Platelet adhesion is also inhibited by prostacyclin, but the concentration required is about ten times greater than the concentration that inhibits platelet aggregation. In 1977 two groups showed that the inhibitory effect of prostacyclin on aggregation was due to its ability to raise the concentration of cyclic AMP in platelets[291,292].

Cyclic AMP

Several drugs that inhibit phosphodiesterase and hence maintain levels of cyclic AMP in platelets were investigated before their mechanism of action was understood. Dipyridamole was already in use in man as a coronary vasodilator before its weak inhibitory effects on platelet aggregation were recognized in 1965 by Emmons and his colleagues[293]. Ardlie and coworkers[294] documented inhibition of platelet aggregation by the methylxanthines, caffeine and theophylline in 1967. Papaverine, dipyridamole and other pyrimido-pyrimidines were shown to enhance the inhibitory effects of adenosine on platelet aggregation[295,296] and block the incorporation of adenosine into platelets. In a major study in 1971, Mills and Smith[297] showed that adenosine increased the concentration of cyclic AMP in platelets and that methylxanthines, papaverine, dipyridamole and other pyrimido-pyrimidines acted as phosphodiesterase inhibitors, preventing the conversion of cyclic AMP to AMP and thus greatly increasing the inhibitory effects of PGE_1 and adenosine on platelet aggregation. Normally, any adenosine formed in plasma is rapidly taken up by red blood cells, but Gresele and his colleagues[298] have suggested that since this uptake is prevented by dipyridamole, the drug may exert most of its inhibitory effect in whole blood by increasing the concentration of adenosine in plasma.

In 1972, Harker and Slichter[299] reported that shortened platelet survival was prolonged by administration of dipyridamole, an effect that was similar to that exerted by sulfinpyrazone which was already being tested in clinical trials for secondary prevention of myocardial infarction. The effect of dipyridamole on platelet survival, the recent new knowledge about its inhibition of platelet aggregation, and the fact that it was already in use in man, led to clinical trials of this drug. However, large clinical trials comparing dipyridamole plus aspirin with aspirin alone for the secondary prevention of coronary artery disease have not shown a significant additional benefit by the inclusion of dipyridamole[269].

Cyclic GMP and nitric oxide

There were reports in 1975 that cyclic GMP increased during platelet aggregation[300], but in 1981, Mellion and colleagues[301] provided evidence for inhibition of aggregation by cyclic GMP when its concentration was increased by nitric oxide, nitroprusside or related vasodilators. It had already been shown that nitroprusside inhibited platelet aggregation[302,303], and that it caused the formation of cyclic GMP in platelets[304]. In 1987 Palmer and coworkers[305] showed that nitric oxide accounted for the activity of EDRF (endothelium-derived relaxing factor) which had been discovered by Furchgott and Zawadzki[306] in 1980. Nitric oxide inhibits platelet adhesion as well as aggregation[307]. In 1993 Radomski and Moncada[308] suggested that the small

Cambridge Books Online © Cambridge University Press, 2009

amount of nitric oxide synthesized by stimulated platelets could account for the early observations of an increase in cyclic GMP caused by platelet aggregating agents.

Signal transduction

Although the results of activation of platelets – adhesion, aggregation, release of granule contents – had been thoroughly studied, it was not until the 1980s that the focus of attention shifted to receptors and the internal signalling events responsible for these platelet functions. The recent history on signal transduction is too voluminous to be reviewed in detail here and can be found in other chapters in this section.

Calcium and phosphoinositides

It was known in the 1960s that external Ca^{2+} is required for platelet aggregation[115,309], but most of the evidence about the role of increases in internal calcium was indirect[310] until 1982 when Rink and colleagues[311] began the use of fluorescent indicators such as Quin 2 developed by Tsien to measure increases in intracellular calcium during platelet activation. The Ca^{2+}-sensitive photoprotein, aequorin, was also used by some investigators[312]. The role of calcium as an intracellular messenger was reviewed by Salzman and Ware[313] in 1989 and Rink and Sage[314] in 1990 (see Chapter 17).

In 1953, Hokin and Hokin[315] had pointed out that phosphoinositide metabolism was involved in stimulus-response coupling in cells, and eight years later Firkin and Williams[316] provided evidence of turnover of platelet phosphinositides in vivo. Using $^{32}PO_4$-labelling, Lloyd and his colleagues in our group showed rapid turnover of phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 4-phosphate (PIP) when platelets were activated with ADP[317] or with collagen or thrombin[318]. In 1975, Michell[319] proposed that hydrolysis of phosphoinositides is responsible for mobilizing internal calcium. This hydrolysis is due to the activation of phospholipase C which acts on PIP_2 to form inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG)[320,321]. IP_3 is responsible for mobilizing calcium from internal stores[322]. (Calcium influx from the external medium also occurs[323].) DAG activates protein kinase C which phosphorylates pleckstrin and myosin light chain kinase[324]. Platelet signalling through the action of tyrosine kinases is reviewed in Chapter 19.

G proteins

It was not until the late 1980s that it became evident that when aggregating agents interacted with their receptors on the platelet membrane, intracellular signalling began with GTP-binding regulatory proteins called G proteins. As this subject has developed, it has been frequently reviewed[325–328] and is discussed in Chapter 14.

Contractile proteins and the cytoskeleton

Bettex-Galland and Lüscher[329] isolated a crude preparation of actin and myosin from human platelets in 1959, providing the first definite evidence that there are contractile proteins in vertebrate nonmuscle cells. They called this actomyosin-like protein 'thrombosthenin'[330]. Interest in it was slow to develop, although its involvement in platelet shape change, spreading, release of granule contents, and clot retraction was assumed[331,332], and has now been established. Detailed studies of the platelet cytoskeleton began in the early 1980s when Phillips and coworkers introduced a technique to obtain a Triton-insoluble filament network from stimulated and unstimulated platelets[333,334] (see Chapter 6).

Conclusion

In the last 50 years there has been an explosive growth in the knowledge of how platelets function, their role in sustaining the integrity of the vascular system, and their response to vessel injury in relation to hemostasis and arterial thrombosis. A better understanding has developed of factors necessary for platelet adhesion to biological surfaces, and how constituents of the vessel wall, particularly collagen, can activate platelets, leading to release of granule contents and formation of thromboxane A_2. Investigations of reactions at the molecular level are providing fresh insights into the basic biological processes through which cells respond to stimuli. The new knowledge has led to substantial advances in our management of disorders of hemostasis and the prevention and treatment of thromboembolism. We still face the challenge that inhibition of platelet function to reduce the risk of arterial thromboembolic problems carries with it the risk of bleeding.

All the chapters in this volume will describe advances built on the work of earlier generations. As Caws[335] wrote in 1969, 'The development of science is a stepwise process; nobody starts from scratch, and nobody gets very far ahead of the rest.'

Cambridge Books Online © Cambridge University Press, 2009

References

1 Donné A. De l'origine des globules du sang, de leur mode de formation et leur fin. *C R Acad Sci* 1842; 14: 366.
2 Wharton-Jones WT. On the state of the blood and blood vessels in inflammation. *Guys Hosp Rep Ser* 1851; 2: 7.
3 Zhan FW. Untersuchungen über Thrombose: Buildung der Thromben. *Virchow's Arch Path Anat* 1875; 62: 81.
4 Bizzozero J. Über einen neuen Formbestandtheil des Blutes und dessen Rolle bei der Thrombose und der Blutgerinnung. *Virchow's Arch Path Anat Physiol Klin Med* 1882; 90: 261–332.
5 Hayem G. Sur le méchanisme de l'arrêt des hémorrhagies. *C R Acad Sci* 1882; 95: 18.
6 Kraus E. Uber purpura. Inaugural dissertation, Heidelberg 1883.
7 Denys J. Étude sur la coagulation du sang dans un cas de purpura avec diminution considerable des plaquettes. *La Cellule* 1887; 3: 445.
8 Lubnitzky S. Die Zusammensetzung des Thrombus in Arterienwunden in den ersten funf Tagen. *Naunyn-Schmeidebergs Arch Pharmacol* 1885; 19: 185.
9 Zucker MB. Platelet agglutination and vasoconstriction as factors in spontaneous hemostasis in normal, thrombocytopenic, heparinized and hypoprothrombinemic rats. *Am J Physiol* 1947; 148: 275–295.
10 Zucker HD. Platelet thrombosis in human hemostasis: a histologic study of skin wounds in normal and purpuric individuals. *Blood* 1949; 4: 631–645.
11 French JE, Macfarlane RG, Sanders AG. The structure of haemostatic plugs and experimental thrombi in small arteries. *Br J Exp Pathol* 1964; 45: 467–474.
12 Tocantins LM. The mammalian blood platelet in health and disease. *Medicine* 1938; 17: 155–260.
13 Macfarlane RG. The theory of blood coagulation. In Biggs R, ed. *Human blood coagulation, haemostasis and thrombosis.* Oxford: Blackwell Scientific Publications 1972; 1–31.
14 Macfarlane RG. An enzyme cascade in the blood clotting mechanism and its function as a biochemical amplifier. *Nature* 1964; 202: 498–499.
15 Duguid JB. Thrombosis as a factor in the pathogenesis of aortic atherosclerosis. *J Pathol Bact* 1948; 60: 57–61.
16 von Rokitansky C. *Handbuch der pathologischen Anatomie.* Vienna: Braumuller u. Siedel; 1841.
17 Fisher CM. Observations of the fundus oculi in transient monocular blindness. *Neurology (Minneapolis)* 1959; 9: 333–347.
18 Danielli JF. Capillary permeability and oedema in perfused frog. *J Physiol* 1940; 98: 109–129.
19 Gimbrone MA Jr, Aster RH, Cotran RS, Corkery J, Jandl JH, Folkman J. Preservation of vascular integrity in organs perfused in vitro with a platelet-rich medium. *Nature* 1969; 221: 33–69.
20 Woods MC, Gamble FN, Furth J, Bigelow RR. Control of the post-irradiation hemorrhagic state by platelet transfusions. *Blood* 1953; 8: 545–553.
21 Cronkite EP, Brecher G. The experimental therapy of the hemorrhagic phase of the radiation syndrome with platelet transfusions. *Acta Radiol* Suppl 1954; 116: 376–380.
22 Cronkite EP, Bond VP, Robertson JS, Paglia DE. The survival, distribution and apparent interaction with capillary endothelium of transfused radiosulfate-labelled platelets in the rat. *J Clin Invest* 1957; 36: 881.
23 Van Horn DL, Johnson SA. The escape of carbon from intact capillaries in experimental thrombocytopenia. *J Lab Clin Med* 1968; 71: 301–311.
24 Gore I, Takada M, Austin J. Ultrastructural basis of experimental thrombocytopenic purpura. *Arch Pathol* 1970; 90: 197–205.
25 Roy AJ, Djerassi I. Effects of platelet transfusions: plug formation and maintenance of vascular integrity. *Proc Soc Exp Biol Med* 1971; 139: 137–142.
26 Kitchens C, Weiss L. Ultrastructural changes of endothelium associated with thrombocytopenia. *Blood* 1975; 46: 567–578.
27 Johnson SA, Balboa RS, Dessel BH, Monto RW, Siegesmund KA, Greenwalt TJ. The mechanism of the endothelial supporting function of intact platelets. *Exp Molec Pathol* 1964; 3: 115–127.
28 Wojcik JD, Van Horn DL, Webber AJ, Johnson SA. Mechanism whereby platelets support the endothelium. *Transfusion* 1969; 9: 324–335.
29 Jaffe E. Endothelial cell structure and function. In Hoffman R, Benz EJ, Shattil S, Furie B, Cohen HJ, eds. *Hematology. Basic principles and practice.* New York: Churchill Livingstone; 1991: 1198–1213.
30 Bounameaux Y. L'accolement des plaquettes aux fibres sous endothéliales. *C R Séances Soc Biol* 1959; 153: 865–867.
31 Kjaerheim A, Hovig T. The ultrastructure of haemostatic blood platelet plugs in rabbit mesenterium. *Thromb Diath Haemorrh* 1962; 7: 1–15.
32 Zucker MB, Borrelli J. Platelet clumping produced by connective tissue suspensions and by collagen. *Proc Soc Exp Biol Med* 1962; 109: 779–787.
33 Hovig T. Release of a platelet aggregating substance (adenosine diphosphate) from rabbit blood platelets induced by saline 'extract' of tendons. *Thromb Diath Haemorrh* 1963; 9: 248–263.
34 Evans G, Nishizawa EE, Packham MA, Mustard JF. The effect of acetylsalicylic acid (aspirin) on platelet function. *Blood* 1967; 30: 550.
35 Evans G, Packham MA, Nishizawa EE, Mustard JF, Murphy EA. The effect of acetylsalicylic acid on platelet function. *J Exp Med* 1968; 128: 877–894.
36 Weiss HJ, Aledort LM. Impaired platelet–connective-tissue reaction in man after aspirin ingestion. *Lancet* 1967; ii: 495–497.
37 Smith JB, Ingerman C, Kocsis JJ, Silver MJ. Formation of an intermediate in prostaglandin biosynthesis and its association with the platelet release reaction. *J Clin Invest* 1974; 53: 1468–1472.
38 Hamberg M, Svensson J, Samuelsson B. Thromboxanes. A

Cambridge Books Online © Cambridge University Press, 2009

new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc Natl Acad Sci USA* 1975; 72: 2994–2998.

39 Hovig T, Rowsell HC, Dodds WJ, Jørgensen L, Mustard JF. Experimental hemostasis in normal dogs and dogs with congenital disorders of blood coagulation. *Blood* 1967; 30: 636–668.

40 Jørgensen L, Borchgrevink CF. The haemostatic mechanisms in patients with haemorrhagic diseases. A histological study of wounds made for primary and secondary bleeding time tests. *Acta Pathol Microbiol Scand* 1964; 60: 55–82.

41 Groves HM, Kinlough-Rathbone RL, Richardson M, Moore S, Mustard JF. Platelet interaction with damaged rabbit aorta. *Lab Invest* 1979; 40: 194–200.

42 Rowntree LG, Shionoya T. Studies in experimental extracorporeal thrombosis. I. A method for the direct observation of extracorporeal thrombus formation. *J Exp Med* 1927; 46: 7–12.

43 Poole JCF, Sanders AG, Florey HW. The regeneration of aortic endothelium. *J Pathol Bacteriol* 1958; 75: 133–143.

44 Williams AW, Montgomery GL. Chemical injury of arteries. *J Pathol Bacteriol* 1959; 77: 63–69.

45 Cotton RE, Harwood TR, Wartman WB. Regeneration of aortic endothelium. *J Pathol Bacteriol* 1961; 81: 175–180.

46 Mustard JF, Rowsell HC, Murphy EA, Downie HG. Intimal thrombosis in atherosclerosis. In: Jones RJ, ed. *Evolution of the atherosclerotic plaque*, Chicago: University of Chicago Press; 1964: 183–203.

47 Baumgartner HR. The role of blood flow in platelet adhesion, fibrin deposition and formation of mural thrombi. *Microvasc Res* 1973; 5: 167–179.

48 Baumgartner HR, Muggli R, Tschopp TB, Turitto VT. Platelet adhesion, release and aggregation in flowing blood: Effects of surface properties and platelet function. *Thromb Haemost* 1976; 35: 124–138.

49 Tschopp TB, Weiss HJ, Baumgartner HR. Decreased adhesion of platelets to subendothelium in von Willebrand's disease. *J Lab Clin Med* 1974; 83: 296–300.

50 Weiss HJ, Tschopp TB, Baumgartner HR, Sussman II, Johnson MM, Egan JJ. Decreased adhesion of giant (Bernard–Soulier) platelets to subendothelium. Further implications on the role of von Willebrand factor in hemostasis. *Am J Med* 1974; 57: 920–925.

51 Baumgartner HR, Tschopp TB, Weiss HJ. Platelet interaction with collagen fibrils in flowing blood. II. Impaired adhesion-aggregation in bleeding disorders. A comparison with subendothelium. *Thromb Haemost* 1977; 37: 17–28.

52 Legrand YJ, Rodriguez-Zeballos A, Kartalis G, Fauvel F, Caen JP. Adsorption of factor VIII antigen-activity complex by collagen. *Thromb Res* 1978; 13: 909–911.

53 Sakariassen KS, Bolhuis PA, Sixma JJ. Human blood platelet adhesion to artery subendothelium is mediated by factor VIII-von Willebrand factor bound to the subendothelium. *Nature* 1979; 279: 636–638.

54 Nyman D. von Willebrand factor dependent platelet aggregation and adsorption of factor VIII related antigen by collagen. *Thromb Res* 1980; 17: 209–214.

55 Fuster V, Bowie EJW, Lewis JC, Fass DN, Owen CA Jr, Brown AL. Arteriosclerosis in pigs with von Willebrand's disease: spontaneous and high cholesterol diet-induced arteriosclerosis. *J Clin Invest* 1978; 61: 722–730.

56 Virchow R. *Phlogose und Thrombose im Gefässystem: Gesammelte abhandlung zur Wissenschaftlichen Medicin.* Frankfurt: Staatsdruckerei 1856.

57 Welch WH. The structure of white thrombi. *Trans Pathol Soc Philadelphia* 1887; 13: 25.

58 Morgan AD. *The pathogenesis of coronary occlusion.* Oxford: Blackwell 1956.

59 More RH, Haust MD. The role of thrombosis in occlusive disease of coronary arteries. In: MacMillan RL, Mustard JF, eds. *Anticoagulants and Fibrinolysins*, Philadelphia: Lea and Febiger; 1961: 143–153.

60 French JE. The structure of natural and experimental thrombi. *Ann Roy Coll Surg Eng* 1965; 36: 191–200.

61 Murphy EA, Rowsell HC, Downie HG, Robinson GA, Mustard JF. Encrustation and atherosclerosis: the analogy between early in vivo lesions and deposits which occur in extracorporeal circulations. *Can Med Assoc J* 1962; 87: 259–274.

62 Mustard JF, Murphy EA, Rowsell HC, Downie HG. Factors influencing thrombus formation in vivo. *Am J Med* 1962; 33: 621–647.

63 Jørgensen L, Packham MA, Rowsell HC, Mustard JF. Deposition of formed elements of blood on the intima and signs of intimal injury in the aorta of rabbit, pig and man. *Lab Invest* 1972; 27: 341–350.

64 Moore S. Thromboatherosclerosis in normolipemic rabbits: a result of continued endothelial damage. *Lab Invest* 1973; 29: 478–487.

65 Moore S, Friedman RJ, Singal DP, Gauldie J, Blajchman MA, Roberts RS. Inhibition of injury-induced thromboatherosclerotic lesions by antiplatelet serum in rabbits. *Thromb Diath Haemorrh* 1976; 35: 70–81.

66 Ross R, Glomset J, Kariya B, Harker L. A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. *Proc Natl Acad Sci USA* 1974; 71: 1207–1210.

67 Jørgensen L, Rowsell, HC, Hovig T, Mustard JF. Resolution and organization of platelet-rich mural thrombi in carotid arteries of swine. *Am J Pathol* 1967; 51: 681–719.

68 Moore S, Mersereau WA. Platelet embolism and renal ischemia. *J Pathol Bacteriol* 1965; 90: 579–588.

69 Jørgensen L, Rowsell HC, Hovig T, Glynn MF, Mustard JF. Adenosine diphosphate-induced platelet aggregation and myocardial infarction in swine. *Lab Invest* 1967; 17: 616–644.

70 Haerem JW. Platelet aggregates in intramyocardial vessels of patients dying suddenly and unexpectedly of coronary artery disease. *Atherosclerosis* 1972; 15: 199–213.

71 Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993; 362: 801–809.

72 Bornfeldt KE, Raines EW, Graves LM, Skinner MP, Krebs EG,

Cambridge Books Online © Cambridge University Press, 2009

Ross R. Platelet-derived growth factor. Distinct signal transduction pathways associated with migration versus proliferation. *Ann New York Acad Sci* 1995; 766: 416–430.
73 Fuster V, Fallon JT, Badimon JJ, Nemerson Y. The unstable atherosclerotic plaque: Clinical significance and therapeutic intervention. *Thromb Haemost* 1997; 78: 247–255.
74 Spaet TH. Platelets: The blood dust. In: Wintrobe MM, ed. *Blood, pure and eloquent. A story of discovery, of people, and of ideas.* New York: McGraw Hill; 1980: 549–571.
75 Hayem G. Recherches sur l'évolution des hématies dans le sang de l'homme et des vertébrés. *Arch Physiol Norm Pathol* 1878; 5: 692–734.
76 Le Sourd L, Pagniez P. Du rôle des hématoblastes dans la retraction du caillot. *C R Soc Biol (Paris)* 1906; 61: 109–111.
77 Budtz-Olsen OE. *Clot retraction*. Oxford: Oxford University Press; 1951: 1–2.
78 Øllgaard E. Clot retraction as a quantitative test of the function and agglutinability of the blood platelets. *Acta Haematol* 1951; 6: 220–230.
79 Bettex-Galland M, Lüscher EF. Studies on the metabolism of human blood platelets in relation to clot retraction. *Thromb Diath Haemorrh* 1960; 4: 178–195
80 Wright JH. The origin and nature of the blood plates. *Boston Med Surg J* 1906; 154: 643–645.
81 Wright JH. The histogenesis of the blood platelets. *J Morphol* 1910; 21: 263–278.
82 Humphrey JH. Origin of blood platelets. Nature 1955; 176: 38.
83 Kinoshita R, Ohno S. Biodynamics of thrombopoiesis. In *Blood platelets. Henry Ford Hospital International Symposium* 1960. Boston: Little, Brown & Co., 1961; pp. 611–616.
84 Kaushansky K. Thrombopoietin: from theory to reality. *Thromb Haemost* 1999; 82: 312–317.
85 Duke WW. The rate of regeneration of blood platelets. *J Exp Med* 1911; 14: 265–273.
86 Hirsch EO, Gardner FH. The lifespan of transfused human platelets. *J Clin Invest* 1951; 30: 649–650.
87 Stefanini M, Chatterjea JB, Dameshek W. Rate of platelet survival in thrombocytopenia. *J Clin Invest* 1951; 30: 676.
88 Stuart MJ, Murphy S, Oski FA. A simple nonradioisotope technique for the determination of platelet life-span. *N Engl J Med* 1975; 292: 1310–1313.
89 Aas KA, Gardner FH. Survival of blood platelets labeled with chromium^{51}. *J Clin Invest* 1958; 37: 1257–1268.
90 Thakur ML, Welch MJ, Joist JH, Coleman RE. Indium-111-labeled platelets: studies on preparation and evaluation of in vitro and in vivo functions. *Thromb Res* 1976; 9: 345–357.
91 Mustard JF, Rowsell HC, Murphy EA. Platelet economy (platelet survival and turnover). *Br J Haematol* 1966; 12: 1–24.
92 Reimers H-J, Kinlough-Rathbone RL, Cazenave J-P et al. In vitro and in vivo functions of thrombin-treated platelets. *Thromb Haemost* 1976; 35: 151–166.
93 Greenberg JP, Packham MA, Guccione MA, Rand ML, Reimers H-J, Mustard JF. Survival of rabbit platelets treated in vitro with chymotrypsin, plasmin, trypsin or neuraminidase. *Blood* 1979; 53: 916–927.
94 Packham MA, Mustard JF. Normal and abnormal platelet activity. In: Lasslo A ed. *Blood platelet function and medicinal chemistry*. New York: Elsevier Biomedical; 1984: 61–128.
95 Shulman NR, Jordan JV Jr. Platelet kinetics. In: Colman RW, Hirsh J, Marder VJ, Salzman EW, eds. *Hemostasis and thrombosis: basic principles and clinical practice*, 2nd ed. Philadelphia: J.B. Lippincott; 1987: 431–451.
96 Murphy EA, Francis ME. The estimation of blood platelet survival. II. The multiple hit model. *Thromb Diath Haemorrh* 1971; 25: 53–80.
97 Panel on Diagnostic Applications of Radioisotopes in Hematology, International Committee for Standardization in Hematology. Recommended methods for radioisotope platelet survival studies. *Blood* 1977; 50: 1137–1144.
98 Dennis JB. *Phil Trans Royal Soc Lond* 1665–1666; 1: 617. 1667; 2: 453. 1668; 3: 710. (Cited by Cronkite EP, Jackson DP. In *Progress in hematology* 1959; 2: 239–257.)
99 Aster RH, Jandl JH. Platelet sequestration in man. I. Methods. *J Clin Invest* 1964; 43: 843–855.
100 Slichter SJ. Platelet transfusions a constantly evolving therapy. *Thromb Haemost* 1991; 66: 178–188.
101 Ardlie NG, Packham MA, Mustard JF. Adenosine diphosphate-induced platelet aggregation in suspensions of washed rabbit platelets. *Br J Haematol* 1970; 19: 7–17.
102 Mustard JF, Perry DW, Ardlie NG, Packham MA. Preparation of suspensions of washed platelets from humans. *Br J Haematol* 1972; 22: 193–204.
103 Mustard JF, Kinlough-Rathbone RL, Packham MA. Isolation of human platelets from plasma by centrifugation and washing. *Methods Enzymol* 1989; 169: 3–11.
104 Walsh PN. Albumin density gradient separation and washing of platelets and the study of platelet coagulant activities. *Br J Haematol* 1972; 22: 205–217.
105 Tangen O, Berman HJ, Marfey P. Gel filtration. A new technique for separation of blood platelets from plasma. *Thromb Diath Haemorrh* 1971; 25: 268–278.
106 Lages B, Scrutton MC, Holmsen H. Studies on gel filtered platelets. Isolation and characterization in a medium containing no added Ca^{2+}, Mg^{2+}, or K^{+}. *J Lab Clin Med* 1975; 85: 811–825.
107 Timmons S, Hawiger J. Isolation of human platelets by albumin gradient and gel filtration. *Methods Enzymol* 1989; 169: 11–21.
108 Born GVR. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature* 1962; 194: 927–929.
109 O'Brien JR. Platelet aggregation. II. Some results from a new method of study. *J Clin Pathol* 1962; 15: 452.
110 Eberth JC, Schimmelbusch C. *Die Thrombose nach Versuchen u. Leichenbefunden*. Stuttgart, Germany: Ferdinand Enke; 1888.
111 Wright JH, Minot GR. The viscous metamorphosis of the blood platelets. *J Exp Med* 1917; 26: 395–409.
112 Apitz K. Ueber Profibrin. IV. Die Agglutination von Blutplättchen durch Profibrin. *Z Gesamte Exp Med* 1939; 105: 89–94.

Cambridge Books Online © Cambridge University Press, 2009

113 Pinniger JL, Prunty FTG. Some observations on the blood-clotting mechanism: role of fibrinogen and platelets with reference to a case of congenital afibrinogenemia. *Br J Exp Pathol* 1946; 27: 200–210.
114 Gaarder A, Jonsen J, Laland S, Hellem AJ, Owren PA. Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. *Nature* 1961; 192: 531–532.
115 Grette K. Studies on the mechanism of thrombin-catalyzed hemostatic reactions in blood platelets. *Acta Physiol Scand* 1962; 56 (suppl 195): 5–93.
116 Haslam RJ. Role of adenosine diphosphate in the aggregation of human platelets by thrombin and by fatty acids. *Nature* 1964; 202: 765–768.
117 Kinlough-Rathbone RL, Packham MA, Reimers H-J, Cazenave J-P, Mustard JF. Mechanisms of platelet shape change, aggregation and release induced by collagen, thrombin or A23,187. *J Lab Clin Med* 1977; 90: 707–719.
118 Vargaftig BB, Chignard M, Benveniste J. Present concepts on the mechanisms of platelet aggregation. *Biochem Pharmacol* 1981; 30: 263–271.
119 Marcus AJ, Safier LB, Ullman HL et al. Effects of acetyl glyceryl ether phosphorylcholine on human platelet function in vitro. *Blood* 1981; 58: 1027–1031.
120 Sturk A, Schaap MCL, ten Cate JW et al. Platelet activating factor: Mediator of the third pathway of platelet aggregation? A study in three patients with deficient platelet-activating factor synthesis. *J Clin Invest* 1987; 79: 344–350.
121 Vu T-KH, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* 1991; 64: 1057–1068.
122 Kinlough-Rathbone RL, Rand ML, Packham MA. Rabbit and rat platelets do not respond to thrombin receptor peptides that activate human platelets. *Blood* 1993; 82: 103–106.
123 Connelly TM, Condra C, Feng D-M et al. Species variability in platelet and other cellular responsiveness to thrombin receptor-derived peptides. *Thromb Haemost* 1994; 72: 627–633.
124 Coughlin SR. Protease-activated receptors and platelet function. *Thromb Haemost* 1999; 82: 353–356.
125 Hamberg M, Svensson J, Wakabayashi T, Samuelsson B. Isolation and structure of two prostaglandin endoperoxides that cause platelet aggregation. *Proc Natl Acad Sci USA* 1974; 71: 345–349.
126 Nieuwenhuis HK, Sakariassen KS, Houdijk WPM, Nievelstein PFEM, Sixma JJ. Deficiency of platelet membrane Ia associated with a decreased platelet adhesion to the subendothelium: a defect in platelet spreading. *Blood* 1986; 68: 692–695.
127 Sugiyama T, Okuma M, Ushikubi, F, Sensaki S, Kanaji K, Uchino H. A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia. *Blood* 1987; 69: 1712–1720.
128 Daniel JL, Dangelmaier C, Strouse R, Smith JB. Collagen induces normal signal transduction in platelets deficient in CD36 (platelet glycoprotein IV). *Thromb Haemost* 1994; 71: 353–356.
129 Nurden AT. Inherited abnormalities of platelets. *Thromb Haemost* 1999; 82: 468–480.
130 Watson SP, Berlanga O, Best D, Frampton J. Update on collagen receptor interactions in platelets: is the two-state model still valid? *Platelets* 2000; 11: 252–258.
131 Hellem AJ. The adhesiveness of human blood platelets in vitro. *Scand J Clin Lab Invest* 1960; 12 (Suppl 51); 1–117.
132 Øllgaard E. Macroscopic studies of platelet aggregation. Nature of an aggregating factor in red blood cells and platelets. *Thromb Diath Haemorrh* 1961; 6: 86–97.
133 Born GVR, Cross MJ. Effect of adenosine diphosphate on the concentration of platelets in circulating blood. *Nature* 1963; 197: 974–976.
134 Constantine JW. Aggregation of guinea-pig platelets by adenosine diphosphate. *Nature* 1966; 164: 162–164.
135 MacMillan DC. Secondary clumping effect in human citrated platelet-rich plasma produced by adenosine diphosphate and adrenaline. *Nature* 1966; 211: 140–144.
136 Zucker MB, Peterson J. Serotonin, platelet factor 3 activity, and platelet-aggregating agent released by adenosine diphosphate. *Blood* 1967; 30: 556.
137 Mills DCB, Robb IA, Roberts GCK. The release of nucleotides, 5-hydroxytryptamine and enzymes from human blood platelets during aggregation. *J Physiol* 1968; 195: 715–729.
138 Packham MA, Kinlough-Rathbone RL, Mustard JF. Thromboxane A_2 causes feedback amplification involving extensive thromboxane A_2 formation upon close contact of human platelets in media with a low concentration of ionized calcium. *Blood* 1987; 70: 647–651.
139 Davey MG, Lander H. Effect of adenosine diphosphate on circulating platelets in man. *Nature* 1964; 201: 1037–1039.
140 Mustard JF, Packham MA. Factors influencing platelet function: adhesion, release, and aggregation. *Pharmacol Rev* 1970; 22: 97–187.
141 Mustard JF, Perry DW, Kinlough-Rathbone RL, Packham MA. Factors responsible for ADP-induced release reaction of human platelets. *Am J Physiol* 1975; 228: 1757–1765.
142 Lages B, Weiss HJ. Dependence of human platelet functional responses on divalent cations: Aggregation and secretion in heparin- and hirudin-anticoagulated platelet-rich plasma and the effects of chelating agents. *Thromb Haemost* 1981; 45: 173–179.
143 Packham MA, Bryant NL, Guccione MA, Kinlough-Rathbone RL, Mustard JF. Effect of the concentration of Ca^{2+} in the suspending medium on the responses of human and rabbit platelets to aggregating agents. *Thromb Haemost* 1989; 62: 968–976.
144 Cole B, Robison GA, Hartmann RC. Studies on the role of cyclic AMP in platelet function. *Ann NY Acad Sci* 1971; 185: 477–487.
145 Mills DCB, Smith JB. The control of platelet responsiveness by agents that influence cyclic AMP metabolism. *Ann NY Acad Sci* 1972; 201: 391–399.
146 Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB. Molecular basis for ADP-induced platelet activation. I. Evidence for

Cambridge Books Online © Cambridge University Press, 2009

three distinct ADP receptors on human platelets. *J Biol Chem* 1998; 273: 2024–2029.
147 Maffrand JP, Eloy F. Synthèse de thiénopyridines et de furopyridines d'intérêt thérapeutique. *Eur J Med Chem* 1974; 9: 483.
148 Thebault JJ, Blatrix CE, Blanchard JF, Panak EA. Effects of ticlopidine, a new platelet aggregation inhibitor in man. *Clin Pharmacol Ther* 1975; 18: 485–490.
149 Saltiel E, Ward A. Ticlopidine: a review of its pharmacodynamic and pharmacokinetic properties and therapeutic efficiency in platelet-dependent disease states. *Drugs* 1987; 34: 222–262.
150 Defreyn G, Gachet C, Savi P, Driot F, Cazenave JP, Maffrand JP. Ticlopidine and clopidogrel (SR 25990C) selectively neutralize ADP inhibition of PGE_1-activated platelet adenylate cyclase in rats and rabbits. *Thromb Haemost* 1991; 65: 186–190.
151 Hechler B, Eckly A, Ohlmann P, Cazenave J-P, Gachet C. The P2Y1 receptor, necessary but not sufficient to support full ADP-induced platelet aggregation, is not the target of the drug clopidogrel. *Br J Haematol* 1998; 103: 858–866.
152 Geiger J, Brich J, Hönig-Liedl P et al. Specific impairment of human platelet $P2Y_{AC}$ ADP receptor-mediated signalling by the antiplatelet drug clopidogrel. *Arterioscl Thromb Vasc Biol* 1999; 19: 2007–2011.
153 Savi P, Pereillo JM, Uzabiaga MF et al. Identification and biological activity of the active metabolite of clopidogrel. *Thromb Haemost* 2000; 84: 891–896.
154 Haas WK, Easton JD, Adams HP Jr, Pryse-Phillips W, Molony BA, Anderson S. A randomized trial comparing ticlopidine hydrochloride with aspirin for prevention of stroke in high-risk patients. Ticlopidine Aspirin Stroke Study Group. *N Engl J Med* 1989; 321: 501–507.
155 Gent M, Blakely JA, Easton JD et al. The Canadian–American Ticlopidine Study (CATS) in thromboembolic stroke. *Lancet* 1989; i: 1215–1220.
156 Caprie Steering Committee. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). *Lancet* 1996; 348: 1329–1339.
157 Cattaneo M, Lecchi A, Randi AM, McGregor JL, Mannucci PM. Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. *Blood* 1992; 80: 2787–2796.
158 Silver MJ, Smith JB, Ingerman C, Kocsis JJ. Arachidonic acid-induced human platelet aggregation and prostaglandin formation. *Prostaglandins* 1973; 4: 863–875.
159 Rand M, Reid G. Source of serotonin in serum. *Nature* 1951; 168: 385.
160 Bigelow FS. Serotonin activity in blood. Measurements in normal subjects and in patients with thrombocythemia hemorrhagica, and other hemorrhagic states. *J Lab Clin Med* 1954; 43: 759–773.
161 Zucker MB, Borrelli J. Quantity, assay and release of serotonin in human platelets. *J Appl Physiol* 1955; 7: 425–431.
162 Humphrey JH, Toh CC. Absorption of serotonin (5-hydroxytryptamine) and histamine by dog platelets. *J Physiol* 1954; 124: 300–304.
163 Born GVR, Gillson RE. Studies on the uptake of 5-hydroxytryptamine by blood platelets. *J Physiol* 1959; 146: 472–491.
164 Tranzer JP, Da Prada M, Pletscher A. Ultrastructural localization of 5-hydroxytryptamine in blood platelets. *Nature* 1966; 212: 1574–1575.
165 Holmsen H, Setkowsky CA, Day HJ. Short communication: possible association of newly absorbed serotonin with non-metabolic, granule-located adenine nucleotides in human blood platelets. *Blood* 1975; 45: 413–416.
166 Mitchell JRA, Sharp AA. Platelet clumping in vitro. *Br J Haematol* 1964; 10: 78–93.
167 Baumgartner HR, Born GVR. Effects of 5-hydroxytryptamine on platelet aggregation. *Nature* 1968; 218: 137–141.
168 Da Prada M, Richards JG, Kettler R. Amine storage organelles in platelets. In: Gordon JL, ed. *Platelets in biology and pathology* 2. Amsterdam: Elsevier; 1981: 105–145.
169 O'Brien JR. Some effects of adrenaline and anti-adrenaline compounds on platelets in vitro and in vivo. *Nature* 1963; 200: 763–764.
170 Haslam RJ. Mechanisms of blood platelet aggregation. In: Johnson SA, Seegers WH, eds. *Physiology of hemostasis and thrombosis*. Springfield, IL: Charles C. Thomas; 1967: 88–112.
171 Ardlie NG, Glew G, Schwartz CJ. Influence of catecholamines on nucleotide-induced platelet aggregation. *Nature* 1966; 212: 415–417.
172 Mills DCB, Roberts GCK. Effects of adrenaline on human blood platelets. *J Physiol* 1967; 193: 443–453.
173 Lalau Keraly C, Kinlough-Rathbone RL, Packham MA, Suzuki H, Mustard JF. Conditions affecting the responses of human platelets to epinephrine. *Thromb Haemost* 1988; 60: 209–216.
174 Lanza F, Beretz A, Stierlé A, Hanau D, Kubina M, Cazenave J-P. Epinephrine potentiates human platelet activation but is not an aggregating agent. *Am J Physiol* 1988; 255: H1276–H1288.
175 Born GVR. The uptake of adrenaline by human platelets. *Naunyn Schmiedebergs Arch Pharmacol* 1968; 259: 155–156.
176 Robison GA, Arnold A, Hartmann RC. Divergent effects of epinephrine and prostaglandin E_1 on the level of cyclic AMP in human blood platelets. *Pharmacol Res Commun* 1969; 1: 325–332.
177 Benveniste J, Henson PM, Cochrane CG. Leucocyte-dependent histamine release from rabbit platelets. *J Exp Med* 1972; 136: 1356–1376.
178 Demopoulos CA, Pinckard RN, Hanahan DJ. Platelet-activating factor. Evidence for 1-o-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine as the active component (a new class of lipid chemical mediators). *J Biol Chem* 1979; 254: 9355–9358.
179 McManus LM, Hanahan DJ, Pinckard RN. Human platelet stimulation by acetyl glyceryl ether phosphorylcholine. *J Clin Invest* 1981; 67: 903–906.
180 Izumi T, Shimizu T. Platelet-activating factor receptor: Gene expression and signal transduction. *Biochim Biophys Acta* 1997; 1259: 317–333.
181 Gangarosa EJ, Johnson TR, Ramos HS. Ristocetin-induced

Cambridge Books Online © Cambridge University Press, 2009

thrombocytopenia: site and mechanism of action. *Arch Intern Med* 1960; 105: 83–89.

182 Howard MA, Firkin BG. Ristocetin – a new tool in the investigation of platelet aggregation. *Thromb Diath Haemorrh* 1971; 26: 362–369.

183 Brinkhous KM, Read MS. Fixation of platelets and platelet agglutination tests. *Methods Enzymol* 1989; 169: 149–163.

184 Bernard J, Soulier JP. Sur une nouvelle variété de dystrophie thrombocytaire-hémorragipare congénitale. *Semin Hôp Paris* 1948; 24: 3217–3223.

185 Howard MA, Hutton RA, Hardisty RM. Hereditary giant platelet syndrome: a disorder of a new aspect of platelet function. *Br Med J* 1973; 4: 586–588.

186 Caen JP, Levy-Toledano S. Interaction between platelets and von Willebrand factor provides a new scheme for primary hemostasis. *Nature New Biol* 1973; 244: 159–160.

187 Nurden AT, Caen JP. Specific roles for platelet surface glycoproteins in platelet function. *Nature* 1975; 255: 720–722.

188 López JA, Andrews RK, Afshar-Khargan V, Berndt MC. Bernard–Soulier Syndrome. *Blood* 1998; 91: 4397–4418.

189 Cross MJ. Effect of fibrinogen on the aggregation of platelets by adenosine diphosphate. *Thromb Diath Haemorrh* 1964; 12: 524–527.

190 MacLean JR, Maxwell RE, Hertler D. Fibrinogen and adenosine diphosphate-induced aggregation of platelets. *Nature* 1964; 202: 605–606.

191 Mustard JF, Packham MA, Kinlough-Rathbone RL, Perry DW, Regoeczi E. Fibrinogen and ADP-induced aggregation. *Blood* 1978; 52: 453–466.

192 Marguerie GA, Plow EF, Edgington TS. Human platelets possess an inducible and saturable receptor specific for fibrinogen. *J Biol Chem* 1979; 254: 5357–5363.

193 Bennett JS, Vilaire G. Exposure of platelet fibrinogen receptors by ADP and epinephrine. *J Clin Invest* 1979; 64: 1393–1401.

194 Peerschke EI, Grant RA, Zucker MB. Relationship between aggregation and binding of ^{125}I-fibrinogen and ^{45}calcium to human platelets. *Thromb Haemost* 1979; 42: 358.

195 Mustard JF, Kinlough-Rathbone RL, Packham MA, Perry DW, Harfenist EJ, Pai KRM. Comparison of fibrinogen association with normal and thrombasthenic platelets on exposure to ADP or chymotrypsin. *Blood* 1979; 54: 987–993.

196 Nurden AT, Caen JP. An abnormal glycoprotein pattern in 3 cases of Glanzmann's thrombasthenia. *Br J Haematol* 1974; 28: 253–260.

197 Kunicki TJ, Pidard D, Rosa J-P, Nurden AT. The formation of Ca^{++}-dependent complexes of platelet membrane glycoproteins IIb and IIIa in solution as determined by crossed immunoelectrophoresis. *Blood* 1981; 58: 268–278.

198 Bennett JS, Vilaire G, Cines DB. Identification of the fibrinogen receptors on human platelets by photoaffinity labeling. *J Biol Chem* 1982; 257: 8049–8054.

199 Pierschbacher MD, Ruoslahti E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* 1984; 309: 30–33.

200 Doolittle RF, Watt KWK, Cottrell BA, Strong DD, Riley M. The amino acid sequence of the alpha-chain of human fibrinogen. *Nature* 1979; 280: 464–468.

201 Titani K, Kumar S, Takio K et al. Amino acid sequence of human von Willebrand factor. *Biochemistry* 1986; 25: 3171–3184.

202 Plow EF, Pierschbacher MD, Ruoslahti E, Marguerie GA, Ginsberg MH. The effect of Arg–Gly–Asp-containing peptides on fibrinogen and von Willebrand factor binding to platelets. *Proc Natl Acad Sci USA* 1985; 82: 8057–8061.

203 Gartner TK, Bennett JS. The tetrapeptide analogue of the cell attachment site of fibronectin inhibits platelet aggregation and fibrinogen binding to activated platelets. *J Biol Chem* 1985; 260: 11891–11894.

204 Haverstick DM, Cowan JF, Yamada KM, Santoro SA. Inhibition of platelet adhesion to fibronectin, fibrinogen, and von Willebrand factor substrates by a synthetic tetrapeptide derived from the cell-binding domain of fibronectin. *Blood* 1985; 66: 946–952.

205 Kloczewiak M, Timmons S, Hawiger J. Localization of a site interacting with human platelet receptor on carboxy-terminal segment of human fibrinogen gamma chain. *Biochem Biophys Res Commun* 1982; 107: 181–187.

206 Farrell DH, Thiagarajan P, Chung DW, Davie EW. Role of fibrinogen α and γ chain sites in platelet aggregation. *Proc Natl Acad Sci USA* 1992; 89: 10729–10732.

207 Farrell DH, Thiagarajan P. Binding of recombinant mutants to platelets. *J Biol Chem* 1994; 269: 226–31.

208 Tcheng JE. Clinical challenges of platelet glycoprotein IIb/IIIa receptor inhibitor therapy. *Am Heart J* 2000; 139: S38–S45.

209 Coller BS, Peerschke EI, Scudder LE, Sullivan CA. A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins IIb and/or IIIa. *J Clin Invest* 1983; 72: 325–338.

210 Coller BS. The role of platelets in arterial thrombosis and the rationale for blockade of platelet GPIIb/IIIa receptors as antithrombotic therapy. *Eur Heart J* 1995; 16 (Suppl L): 11–15.

211 Mustard JF, Packham MA, Kinlough-Rathbone RL. Mechanisms in thrombosis. In: Bloom AL, Thomas DP, eds. *Haemostasis and thrombosis*, 2nd ed. Edinburgh: Churchill Livingstone 1987; 618–650.

212 Zucker MB, Borrelli J. Relationship of some clotting factors to serotonin release from washed platelets. *J Appl Physiol* 1955; 7: 432–442.

213 Humphrey JH, Jaques R. The release of histamine and 5-hydroxytryptamine (serotonin) from platelets by antigen-antibody reactions (in vitro). *J Physiol* 1955; 128: 9–27.

214 Spaet TH, Zucker MB. Mechanism of platelet plug formation and role of adenosine diphosphate. *Am J Physiol* 1964; 206: 1267–1274.

215 Buckingham S, Maynert EW. The release of 5-hydroxytryptamine, potassium and amino acids from platelets. *J Pharmacol Exp Ther* 1964; 143: 332–339.

Cambridge Books Online © Cambridge University Press, 2009

216 Ireland DM. Effect of thrombin on the radioactive nucleotides from human washed platelets. *Biochem* J 1967; 105: 857–867.
217 Holmsen H, Day HJ. Thrombin-induced platelet release reaction and platelet lysosomes. *Nature* 1968; 219: 760–761.
218 Davey MG, Lüscher EF. Release reaction of human platelets induced by thrombin and other agents. *Biochim Biophys Acta* 1968; 165: 490–506.
219 Niewiarowski S, Thomas DP. Platelet factor 4 and adenosine diphosphate release during human platelet aggregation. *Nature* 1969; 222: 1269–1270.
220 Mürer EH. Thrombin induced release of calcium from blood platelets. *Science* 1969; 166: 623.
221 Holmsen H, Day HJ, Stormorken H. The blood platelet release reaction. *Scand J Haematol* 1969 (Suppl); 8: 3–26.
222 Fukami MH, Salganicoff L. Human platelet storage organelles. A review. *Thromb Haemost* 1977; 38: 963–970.
223 Holmsen H, Weiss HJ. Hereditary defect in the platelet release reaction caused by a deficiency in the storage pool of platelet adenine nucleotides. *Br J Haematol* 1970; 19: 643–649.
224 Mustard JF, Movat HZ, MacMorine DRL, Senyi A. Release of permeability factors from the blood platelet. *Proc Soc Exp Biol Med* 1965; 119: 988–991.
225 Hsu-Liu SC, Berman CL, Furie BC, August D, Furie B. A platelet membrane protein expressed during platelet activation. *J Biol Chem* 1984; 259: 9121–9126.
226 McEver RP, Martin MN. A monoclonal antibody to a membrane glycoprotein binds only to activated platelets. *J Biol Chem* 1984; 259: 9799–9804.
227 Berman CL, Yeo EL, Wencel-Drake JD, Furie BC, Ginsberg MH, Furie B. A platelet alpha granule membrane protein that is associated with the plasma membrane after activation. Characterization and subcellular localization of platelet activation-dependent granule-external membrane protein. *J Clin Invest* 1986; 78: 130–137.
228 Michelson AD. Flow cytometry. A clinical test of platelet function. *Blood* 1996; 87: 4925–4936.
229 Larsen E, Celi A, Gilbert GE et al. PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. *Cell* 1989; 59: 305–312.
230 Chargaff E, Bancroft FW, Stanley-Brown M. Studies on the chemistry of blood coagulation. III. The chemical constituents of blood platelets and their role in blood clotting, with remarks on the activation of clotting by lipids. *J Biol Chem* 1936; 116: 237–251.
231 van Crevald S, Paulssen MMP. Significance of clotting factors in blood platelets, in normal and pathological conditions. *Lancet* 1951; ii: 242–244.
232 van Crevald S, Paulssen MM. Isolation and properties of the third clotting factor in blood platelets. *Lancet* 1952; 1: 23–25.
233 Alkjaersig N, Abe T, Seegers WH. Purification and quantitative determination of platelet factor 3. *Am J Physiol* 1955; 181: 304–308.
234 Fantl P, Ward HA. The thromboplastic component of intact blood platelets is present in masked form. *Austr J Exp Biol Med Sci* 1958; 36: 499–504.
235 Hardisty RM, Hutton RA. The kaolin clotting time of platelet-rich plasma: A test of platelet factor-3 availability. *Br J Haematol* 1965; 11: 258–268.
236 Spaet TH, Cintron J. Studies on platelet factor-3 availability. *Br J Haematol* 1965; 11: 269–275.
237 Joist JH, Dolezel G, Lloyd JV, Kinlough-Rathbone RL, Mustard JF. Platelet factor-3 availability and the platelet release reaction. *J Lab Clin Med* 1974; 84: 474–482.
238 Bevers EM, Comfurius P, Van Rijn JLML, Hemker HC, Zwaal RFA. Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. *Eur J Biochem* 1982; 122: 429–436.
239 Bevers EM, Comfurius P, Zwaal RFA. Changes in membrane phospholipid distribution during platelet activation. *Biochim Biophys Acta* 1983; 736: 57–66.
240 Sune A, Bette-Bobillo P, Bienvenüe A, Fellmann P, Devaux PF. Selective outside-inside translocation of aminophospholipids in human platelets. *Biochemistry* 1987; 26: 2972–2978.
241 Comfurius P, Williamson P, Smeets EF, Schlegel RA, Bevers EM, Zwaal RFA. Reconstitution of phospholipid scramblase activity from human blood platelets. *Biochemistry* 1996; 35: 7631–7634.
242 Zwaal RFA, Schroit AJ. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood* 1997; 89: 1121–1132.
243 Zwaal RFA, Comfurius P, Bevers EM. Lipid–protein interactions in blood coagulation. *Biochim Biophys Acta* 1998; 1376: 433–453.
244 Weiss HJ, Vicic WJ, Lages BA, Rogers J. Isolated deficiency of platelet procoagulant activity. *Am J Med* 1979; 67: 206–213.
245 Weiss HJ. Scott syndrome: A disorder of platelet coagulant activity. *Semin Hematol* 1994; 31: 312–319.
246 Wolf P. The nature and significance of platelet products in human plasma. *Br J Haematol* 1967; 13: 269–288.
247 Crawford N. The presence of contractile proteins in platelet microparticles isolated from human and animal platelet-free plasma. *Br J Haematol* 1971; 21: 53–69.
248 Sandberg H, Andersson L-O, Hölund S. Isolation and characterization of lipid-protein particles containing platelet factor 3 released from human platelets. *Biochem J* 1982; 203: 303–311.
249 Sandberg H, Bode AP, Dombrose FA, Hoechli M, Lentz BR. Expression of coagulant activity in human platelets: Release of membranous vesicles providing platelet factor 1 and platelet factor 3. *Thromb Res* 1985; 39: 63–79.
250 Sims PJ, Faioni EM, Wiedmer T, Shattil SJ. Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity. *J Biol Chem* 1988; 263: 18205–18212.
251 Pasquet JM, Dachary-Prigent J, Nurden AT. Microvesicle release is associated with extensive protein tyrosine dephosphorylation in platelets stimulated by A23187 or a mixture of thrombin and collagen. *Biochem J* 1998; 333: 591–599.

Cambridge Books Online © Cambridge University Press, 2009

252 George JN, Pickett EB, Saucerman S et al. Platelet surface glycoproteins. Studies on resting and activated platelets in normal subjects, and observations in patients during adult respiratory distress syndrome and cardiac surgery. *J Clin Invest* 1986; 78: 340–348.
253 Owens MR. The role of platelet microparticles in hemostasis. *Transfusion Med Rev* 1994; 8: 37–44.
254 Beaumont JL, Caen J, Bernard J. Action hemorragipare de l'acide acetyl-salicylique au cours des maladies du sang. *Bull Soc Med Hop Paris* 1955; 71: 1087–1092.
255 Frick PG. Hemorrhagic diathesis with increased capillary fragility caused by salicylate therapy. *Am J Med Sci* 1956; 231: 402–406.
256 Gast LF. Influence of aspirin on haemostatic parameters. *Ann Rheum Dis* 1964; 23: 500–504.
257 Quick AJ. Salicylates and bleeding: The aspirin tolerance test. *Am J Med Sci* 1966; 252: 265–269.
258 Mustard JF, Murphy EA, Ogryzlo MA, Smythe HA. Blood coagulation and platelet economy in subjects with primary gout. *Can Med Assoc J* 1963; 89: 1207–1211.
259 Smythe HA, Ogryzlo MA, Murphy EA, Mustard JF. The effect of sulfinpyrazone (anturan) on platelet economy and blood coagulation in man. *Can Med Assoc J* 1965; 92: 818–821.
260 Packham MA, Warrior ES, Glynn MF, Senyi AS, Mustard JF. Alteration of the response of platelets to surface stimuli by pyrazole compounds. *J Exp Med* 1967; 126: 171–188.
261 Mustard JF, Rowsell HC, Smythe HA, Senyi A, Murphy EA. The effect of sulfinpyrazone on platelet economy and thrombus formation in rabbits. *Blood* 1967; 29: 859–866.
262 Zucker MB, Peterson J. Inhibition of adenosine diphosphate-induced secondary aggregation and other platelet functions by acetylsalicylic acid ingestion. *Proc Soc Exp Biol Med* 1968; 127: 547–551.
263 O'Brien JR. Effects of salicylates on human platelets. *Lancet* 1968; i: 779–783.
264 Al-Mondhiry H, Marcus A, Spaet TH. Acetylation of human platelets by aspirin. *Fed Proc* 1969; 28: 576.
265 Roth GJ, Stanford N, Majerus PW. Acetylation of prostaglandin synthetase by aspirin. *Proc Natl Acad Sci USA* 1975; 72: 3073–3076.
266 Mitchell JA, Warner TD. Cyclo-oxygenase-2; Pharmacology, physiology, biochemistry and relevance to NSAID therapy. *Br J Pharmacol* 1999; 128: 1121–1132.
267 Braunwald E, Friedewald WT, Furberg CD. Proceedings of the workshop on platelet active drugs in the secondary prevention of cardiovascular events. *Circulation* 1980; 62 (Suppl V): V1–V135.
268 Antiplatelet Trialists' Collaboration. Secondary prevention of vascular disease by prolonged antiplatelet treatment. *Br Med J* 1988; 296: 320–331.
269 Hennekens CH, Buring JE, Sandercock P, Collins R, Peto R. Aspirin and other antiplatelet agents in the secondary and primary prevention of cardiovascular disease. *Circulation* 1989; 80: 749–756.
270 Craven LL. Experiences with aspirin (acetylsalicylic acid) in the non-specific prophylaxis of coronary thrombosis. *Mississippi Valley Med J* 1953; 75: 38–44.
271 Craven LL. Prevention of coronary and cerebral thrombosis. *Mississippi Valley Med J* 1956; 78: 213–215.
272 Barnett HJM, Gent M, Sackett DL et al. A randomized trial of aspirin and sulfinpyrazone in threatened stroke. The Canadian Cooperative Study Group. *N Engl J Med* 1978; 299: 53–59.
273 Albers GW, Tijssen JGP. Antiplatelet therapy: New foundations for optimal treatment decisions. *Neurology* 1999; 53 (Suppl 4): S25–S31.
274 Bergström S, Sjövall J. The isolation of prostaglandin. *Acta Chem Scand* 1957; 11: 1086.
275 Kloeze J. Influence of prostaglandins on platelet adhesiveness and platelet aggregation. In Proceedings of the 2nd Nobel Symposium. Bergström S, Samuelsson B, eds. New York: *Interscience* 1967; 241–252.
276 Emmons PR, Hampton JR, Harrison MJG, Honour AJ, Mitchell JRA. Effect of prostaglandin E_1 on platelet behaviour in vitro and in vivo. *Br Med J* 1967; 21: 468–472.
277 Bergström S. Prostaglandins: Members of a new hormonal system. These physiologically very potent compounds of ubiquitous occurrence are formed from essential fatty acids. *Science* 1967; 157: 382–391.
278 Robison GA, Arnold A, Hartmann RC. Divergent effects of epinephrine and prostaglandin E_1 on the level of cyclic AMP in human blood platelets. *Pharmacol Res Commun* 1969; 1: 325–332.
279 Zucker MB. In *The physiology of blood platelets*. Marcus AJ, Zucker MB. New York: Grune and Stratton; 1965: 53.
280 Smith JB, Willis AL. Formation and release of prostaglandins in response to thrombin. *Br J Pharmacol* 1970; 40: 545P.
281 Piper PJ, Vane JR. Release of additional factors in anaphylaxis and its antagonism by antiinflammatory drugs. *Nature* 1969; 223: 29–35.
282 Gryglewski R, Vane JR. The release of prostaglandins and rabbit aorta contracting substance (RCS) from rabbit spleen and its antagonism by anti-inflammatory drugs. *J Pharmacol* 1972; 45: 37–47.
283 Smith JB, Willis AL. Aspirin selectively inhibits prostaglandin production in human platelets. *Nature New Biol* 1971; 231: 235–237.
284 Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature New Biol* 1971; 231: 232–235.
285 Vargaftig BB, Zirnis P. Platelet aggregation induced by arachidonic acid is accompanied by release of potential inflammatory mediators distinct from PGE_2 and $PGF_{2\alpha}$. *Nature New Biol* 1973; 244: 114–116.
286 Willis AL, Vane FM, Kuhn DG, Scott CG, Petrin M. An endoperoxide aggregator (LASS), formed in platelets in response to thrombotic stimuli: Purification, identification and unique biological significance. *Prostaglandins* 1974; 8: 453–507.
287 Hamberg M, Samuelsson S. Prostaglandin endoperoxides.

Cambridge Books Online © Cambridge University Press, 2009

Novel transformations of arachidonic acid in human platelets. *Proc Natl Acad Sci USA* 1974; 71: 3400–3404.
288 Loll PJ, Picot D, Garavito RM. The structural basis of aspirin activity inferred from the crystal structure of inactivated prostaglandin H_2 synthase. *Nat Struct Biol* 1995; 2: 637–643.
289 Moncada S, Gryglewski R, Bunting S, Vane JR. An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature* 1976; 263: 663–665.
290 Higgs EA, Moncada S, Vane JR, Caen JP, Michel H, Tobelem G. Effect of prostacyclin (PGI_2) on platelet adhesion to rabbit arterial subendothelium. *Prostaglandins* 1978; 16: 17–22.
291 Gorman RR, Bunting S, Miller OV. Modulation of human platelet adenylate cyclase by prostacyclin (PGX). *Prostaglandins* 1977; 13: 377–388.
292 Tateson JE, Moncada S, Vane JR. Effects of prostacyclin (PGX) on cyclic AMP concentrations in human platelets. *Prostaglandins* 1977; 13: 389–397.
293 Emmons PR, Harrison MJG, Honour AJ, Mitchell JRA. Effect of dipyridamole on human platelet behaviour. *Lancet* 1965; ii: 603–606.
294 Ardlie NG, Glew G, Schultz BG, Schwartz CJ. Inhibition and reversal of platelet aggregation by methylxanthines. *Thromb Diath Haemorrh* 1967; 18: 670–673.
295 Markwardt F, Barthel W, Glusa E, Hoffmann A. Untersuchung über den Einfluss von Papaverin auf Reaktionen der Blutplättchen. *Naunyn Schmeidebergs Arch Pharmacol* 1967; 257: 420–431.
296 Born GVR, Mills DCB. Potentiation of the inhibitory effect of adenosine on platelet aggregation by drugs that prevent its uptake. *J Physiol* 1969; 202: 41P-2P.
297 Mills DCB, Smith JB. The influence on platelet aggregation of drugs that affect the accumulation of adenosine 3′:5′-cyclic monophosphate in platelets. *Biochem J* 1971; 121: 185–196.
298 Gresele P, Arnout J, Deckmyn H, Vermylen J. Mechanism of the antiplatelet action of dipyridamole in whole blood; Modulation of adenosine concentration and activity. *Thromb Haemost* 1986; 55: 12–18.
299 Harker LA, Slichter SJ. Platelet and fibrinogen consumption in man. *N Engl J Med* 1972; 287: 999–1005.
300 Haslam RJ. Roles of cyclic nucleotides in platelet function. In *Biochemistry and pharmacology of platelets*. Ciba Foundation Symposium 35 (new series). Amsterdam: Elsevier 1975; 121–151.
301 Mellion BT, Ignarro LJ, Ohlstein EH, Pontecorvo EG, Hyman AL, Kadowitz PJ. Evidence for the inhibitory role of guanosine 3′,5′-monophosphate in ADP-induced human platelet aggregation in the presence of nitric oxide and related vasodilators. *Blood* 1981; 57: 946–954.
302 Pfleiderer T. Na-nitroprussid, a very potent platelet disaggregating substance. *Acta Univ Carol [Med Monogr](Praha)* 1972; 53: 247–250.
303 Saxon A, Kattlove HE. Platelet inhibition by sodium nitroprusside, a smooth muscle inhibitor. *Blood* 1976; 47: 957–961.
304 Bohme E, Graf H, Schultz G. Effects of sodium nitroprusside and other smooth muscle relaxants on cyclic GMP formation in smooth muscle and platelets. *Adv Cyclic Nucl Res* 1978; 9: 131–143.
305 Palmer RMJ, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987; 327: 524–526.
306 Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 1980; 288: 373–376.
307 Graaf de JC, Banga JD, Moncada S, Palmer RMJ, de Groot PG, Sixma JJ. Nitric oxide functions as an inhibitor of platelet adhesion under flow conditions. *Circulation* 1992; 85: 2284–2290.
308 Radomski MW, Moncada S. Regulation of vascular homeostasis by nitric oxide. *Thromb Haemost* 1993; 70: 36–41.
309 Skoza L, Zucker MB, Jerushalmy Z, Grant R. Kinetic studies of platelet aggregation induced by adenosine diphosphate and its inhibition by chelating agents, guanidine compounds and adenosine. *Thromb Diath Haemorrh* 1967; 18: 713–775.
310 Holmsen H. Are platelet shape change, aggregation and release reactions tangible manifestations of one basic platelet function? In: Baldini MG, Ebbe S, eds. *Platelets: production, function, transfusion, and storage*. New York: Grune and Stratton; 1974: 207–220.
311 Rink TJ, Smith SW, Tsien RY. Intracellular free calcium in platelet shape change and aggregation. *J Physiol* 1982; 324: 53P–54P.
312 Johnson PC, Ware JA, Cliveden PB, Smith M, Dvorak AM, Salzman EW. Measurement of ionized calcium in blood platelets with the photoprotein aequorin. Comparison with Quin 2. *J Biol Chem* 1985; 260: 2069–2076.
313 Salzman EW, Ware JA. Ionized calcium as an intracellular messenger in blood platelets. In: Coller BS, ed. *Progress in hemostasis and thrombosis*. Vol 9. Philadelphia: W.B. Saunders; 1989: 177–202.
314 Rink TJ, Sage SO. Calcium signalling in human platelets. *Annu Rev Physiol* 1990; 52: 431–449.
315 Hokin MR, Hokin LE. Enzyme secretion and the incorporation of P^{32} into phospholipids of pancreas slices. *J Biol Chem* 1953; 203: 967–977.
316 Firkin BG, Williams WJ. The incorporation of radioactive phosphorus into the phospholipids of human leukemic leukocytes and platelets. *J Clin Invest* 1961; 40: 423–432.
317 Lloyd JV, Nishizawa EE, Haldar J, Mustard JF. Changes in ^{32}P-labelling of platelet phospholipids in response to ADP. *Br J Haematol* 1972; 23: 571–585.
318 Lloyd JV, Mustard JF. Changes in ^{32}P-content of phosphatidic acid and the phosphoinositides of rabbit platelets during aggregation induced by collagen or thrombin. *Br J Haematol* 1974; 26: 243–253.
319 Michell RH. Inositol phospholipids and cell surface receptor function. *Biochim Biophys Acta* 1975; 415: 81–147.
320 Berridge MJ. Inositol trisphosphate and diacylglycerol as second messengers. *Biochem J* 1984; 220: 345–360.
321 Nishizuka Y. Turnover of inositol phospholipids and signal transduction. *Science* 1984; 225: 1365–1370.

Cambridge Books Online © Cambridge University Press, 2009

322 Brass LF, Joseph SK. A role for inositol trisphosphate in intracellular Ca^{2+} mobilization and granule secretion in platelets. *J Biol Chem* 1985; 260: 15172–15179.
323 Hallam TJ, Rink TJ. Agonists stimulate divalent cation channels in the plasma membrane of human platelets. FEBS Lett 1985; 186: 175.
324 Levy-Toledano S, Gallet C, Nadal F, Bryckaert M, Maclouf J, Rosa J-P. Phosphorylation and dephosphorylation mechanisms in platelet function: A tightly regulated balance. *Thromb Haemost* 1997; 78: 226–233.
325 Siess W. Molecular mechanisms of platelet activation. *Physiol Rev* 1989; 69: 58–178.
326 Kroll MH, Schafer. Biochemical mechanisms of platelet activation. *Blood* 1989; 74: 1181–1195.
327 Brass LF, Hoxie JA, Manning DR. Signalling through G proteins and G protein-coupled receptors during platelet activation. *Thromb Haemost* 1993; 70: 217–223.
328 Brass LF, Manning DR, Cichowski K, Abrams CS. Signalling through G proteins in platelets: to the integrins and beyond. *Thromb Haemost* 1997; 78: 581–589.
329 Bettex-Galland M, Lüscher EF. Extraction of an actomyosin-like protein from human platelets. *Nature* 1959; 184: 276–277.
330 Bettex-Galland M, Lüscher EF. Thrombosthenin, the contractile protein from blood platelets and its relation to other contractile proteins. *Adv Prot Chem* 1965; 20: 1–35.
331 Adelstein RS, Pollard TD. Platelet contractile proteins. In: Spaet TH, ed. *Progress in hemostasis and thrombosis*. Vol 4. New York: Grune and Stratton; 1978: 37–58.
332 Lind SE, Stossel TP. The microfilament network of the platelet. In: Spaet TH, ed. *Progress in hemostasis and thrombosis*. Vol 6. New York; Grune and Stratton; 1982: 63–84.
333 Phillips DR, Jennings LK, Edwards HH. Identification of membrane proteins mediating the interaction of human platelets. *J Cell Biol* 1980; 86: 77–86.
334 Jennings LK, Fox JEB, Edwards HH, Phillips DR. Changes in the cytoskeletal structure of human platelets following thrombin activation. *J Biol Chem* 1981; 256: 6927–6932.
335 Caws P. The structure of discovery. *Science* 1969; 166: 1375–1380.

Cambridge Books Online © Cambridge University Press, 2009

2

Production of platelets

Arnaud Drouin and Elisabeth M. Cramer

Institut Cochin Département d'Hématologie and Faculté de Médicine Paris-Ouest, Paris, France

Introduction

Platelets are small anucleated cell fragments, which are essential components of primary haemostasis. They are derived from the cytoplasmic fragmentation of a giant precursor cell, the megakaryocyte (MK). MKs arise and mature in the bone marrow, along with the other blood cell precursors, e.g. granulocytes and erythroblasts. 150 to 400×10^9/l platelets usually circulate within normal human blood with an average lifespan of approximately 10 days. Therefore one would predict that about $15 \times 10^9 - 40 \times 10^9$ platelets have to be produced each day to maintain normal levels. Appropriate platelet production is not only dependent upon a normal rate of thrombopoiesis but also upon the delivery of platelets of the correct size and functionality with normal subcellular organization. Therefore, the qualitative as well as the quantitative aspects of thrombopoiesis will be discussed in this chapter.

Cellular aspect of platelet production

MK localization

Normal human MKs, are mainly located in the bone marrow (Fig. 2.1, see colour plate). They are often gathered into small groups usually up to three, which consist of different cells of various size, ploidy and maturation stages. They are usually located in close proximity to a vascular sinusoid[1]: This is important as MKs and endothelial cells can communicate by many potential pathways: MKs contain mitogenic factors for endothelial cells, e.g. vascular endothelial growth factor (VEGF)[2], whereas endothelial cells express receptors and adhesion molecules which can potentially retain MK close to the bloodstream where future platelets could be delivered[3]. Their accumulation near the sinusoids also potentially represents a physical barrier for other marrow cells migrating into the circulation. However, foreign cells are able to penetrate the MK through the open channels of the demarcation membranes and transmigrate across the whole MK volume without any significant damage to either cell. This natural phenomenon, termed 'emperipolesis', is occasionally observed within normal bone marrow but is distinct from phagocytosis[4]. However, in pathological conditions, e.g. myeloproliferative syndromes with myelofibrosis, the frequency of emperipolesis is not only increased but becomes pathogenic to both cell types with disastrous consequences to the immediate cellular environment[5]. In animal species such as mice and rats but not humans, maturing MKs are equally distributed in the bone marrow and the spleen, and to a lesser extent in the liver[6,7]. In addition, MKs are also observed within the pulmonary circulation of both human and animals[8].

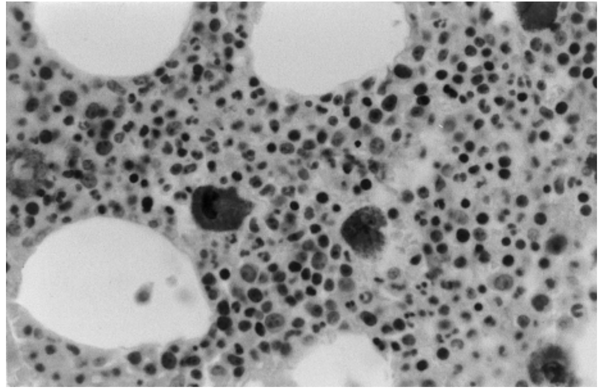
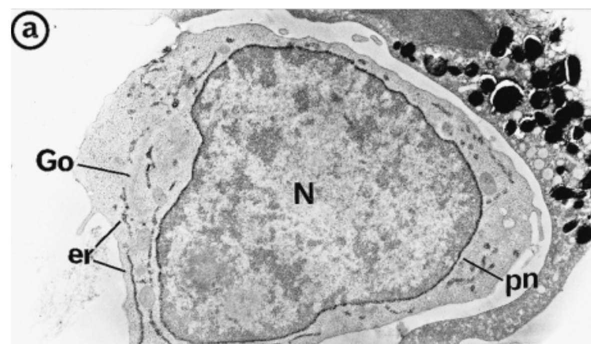


Fig. 2.1 (see also colour plate). Human bone marrow section stained for fibrinogen by the APAAP technique: MKs specifically stain red. They display a large size and polylobulated nucleus, and are quite seldom in the marrow space compared with the other hematopoietic cell lines. They often stand close to a vascular sinusoid.

Remarkably, mature bone marrow MKs are extremely deformable and are not only able to adhere to, but transmigrate across, the sinusoidal endothelial barrier to enter the circulation[9]; the first capillary bed that they encounter is the pulmonary microcirculation in which they can be easily entrapped. Intact, fully mature, large MKs have definitely been recovered in the lung circulation, while large MK naked nuclei can be found downstream from the lungs, in the aorta[10]. These observations, which implicate the lung as a major site of platelet production are discussed in detail within the last section of this chapter.



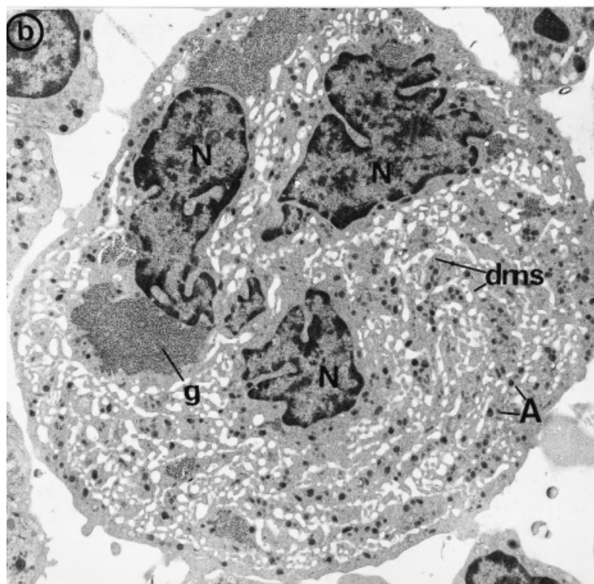


Fig. 2.2. Electron microscopic aspect of MKs at various maturation stages. (*a*) The diploid MK precursor lacks distinctive morphological features, but can be identified based on its content of peroxidase activity revealed by a cytochemical reaction which specifically opacifies the perinuclear cisternae (pn) and endoplasmic reticulum (er). Golgi complex (Go) (apart from an occasional cisternae) and the rare secretion granules are consistently negative. N = nucleus (M × 4367). (*b*) Mature bone marrow MKs are characterized by their large size and multilobed nucleus (N). They contain specific organelles, i.e. numerous α-granules (A) and a well-developed demarcation membrane system (dms) regularly scattered throughout the cytoplasm. This system is formed by the invagination of the plasma membrane, develops extremely rapidly and is the precursor of the platelet membrane systems. g = glycogen (M × 2594).

MK diploid precursor

MKs are derived, together with the other hematopoietic cell lines, from a pluripotent hematopoietic progenitor, which becomes committed to the megakaryocytic lineage that is present in the bone marrow. They can also be recovered from either the peripheral blood or cord blood where they are particulary abundant. During the proliferation stage, the diploid MK progenitors multiply by conventional mitosis. These progenitors are not yet morphologically recognizable and can only be identified through some of their properties obtained in culture[11,12].

The diploid committed precursors have a proliferative capacity but display no distinctive morphological features, and have been characterized based on their functional capacities: BFU (burst forming unit) -MK, the earliest identifiable progenitor, is present in the bone marrow as a CD34+ blast cell which has a proliferative capacity, and gives rise to large colonies of MK (40–500 cells per colony) after 16–20 days of culture. CFU (colony forming unit) -MKs are a heterogeneous population of cells (colony forming cell), and express the CD34 antigen and HLA-DR, and eventually platelet specific glycoproteins (Gp) such as GpIIb/IIIa[13]. Electron microscopy is also a sensitive tool which can be utilized to cytochemically detect the presence of platelet peroxidase (PPO) in the perinuclear cisternae and endoplasmic reticulum; the enzyme is absent from the Golgi apparatus and specific to the human species[14]. In contrast, acetylcholinesterase is a useful marker of the MK lineage of rodents and cats[6,7].

MK maturation and differentiation

Cell maturation and differentiation is a continuous and progressive process. However, three sequential MK maturation stages have been identified according to observations by light microscopy[15].

MK stage I or megakaryoblast is the first morphologically recognizable MK cell. Under the light microscope, it resembles the pluripotent hematopoietic stem cell or hemoblast: high nucleus/cytoplasm ratio, immature thin chromatin, prominent nucleoli, and a small rim of basophilic cytoplasm. The distinctive feature is its large nuclear size, which reflects the fact that its ploidy has increased. At

Cambridge Books Online © Cambridge University Press, 2009

the electron microscope level, the nuclear chromatin is mainly clear, transcriptionally active euchromatin. The cytoplasm is rich in ribosomes undergoing active protein synthesis and responsible for basophilia. A few mitochondria of large size are randomly scattered (Fig. 2.2(*a*)). At the next maturation stage, MK stage II, the nucleus displays a budding shape, having nearly completed its final ploidy maturation, and the cytoplasm extends and becomes deeply basophilic. MK stage III is the platelet forming stage: the cell reaches its maximal size, the nucleus is polylobulated – on squash bone marrow preparations, the number of nuclear lobes appears to be correlated to ploidy – and the cytoplasm becomes progressively azurophilic with a platelet-like red stippling progressively replacing the basophilia.

Ultrastructural examination facilitates the delineation of subcellular events accompanying MK growth and differentiation: the nucleus increases its DNA content and ploidy by a unique phenomenon called endomitosis. In parallel, two specific types of organelles: the demarcation membrane system and the secretion granules develop simultaneously[16].

Nucleus and endomitosis

Nucleus

Upon cell maturation, the nucleus displays several sparse rounded lobes with abundant euchromatin, and conspicuous nucleoli. Its maturation is characterised by constant size increase and segmentation, and by chromatin clumping. Close to the final maturation stage of MK, the structure and texture of the nucleus begin to change. The nuclear lobes tend to elongate and gather together, the chromatin becomes coarser, and long clefts of cytoplasm extend between each lobe. Finally, the nucleus has usually terminated its growth when the main events of the synthesis of cytoplasmic organelles are taking place.

Endomitosis

At the end of the proliferative phase, 2N MK precursors endomitose until a level ranging from 4N to 64N is reached; most cells which give rise to platelets are thought to be 8, 16 or 32N with a modal number of 16N in humans. However, the exact signal(s) which switch the cell from normal mitosis to endomitosis are still unknown. Polyploidization of MK is unique among normal mammalian cells in that multiples of 2N chromosomes are enclosed within a single nucleus and surrounded by a single nuclear membrane. To achieve this, MKs undergo a unique process called endomitosis during which DNA duplicates, followed by nucleus segmentation appropriately, but without apparent nuclear or cytoplasmic division[17,18]. Morphological studies on the structure of MK endomitosis have shown that a multipolar mitotic spindle is formed, with the number of spindle poles in a single MK varying from 4 to 64 N. The nuclear membrane breaks down and disappears during polyploidization as in any classical diploid mitosis. Metaphase occurs and multiple mitotic spindles form with spindle poles symmetrically located at right angles from one another. During anaphase, many bundles of sister chromatids are pulled apart toward the spindle pole they face. However, each pair of centrioles remains closer to each other than in normal cell division and neither telophase, nor cytokinesis (cellular division) follow the unusual truncated anaphase. Eventually, spindles remain shorter than normal ones, connected to each other, a single pole connecting to several of them. In addition, the multiple pole mitotic spindle creates a spherical structure, but does not cross the cell centre and is often located aside within the sphere. Finally, the chromosomes segregate near each pole, forming the nuclear lobes.

Cytoplasmic differentiation

Demarcation membrane system

During the maturation process, a network of smooth membrane channels are formed, i.e. the demarcation membrane system (Fig. 2.2(*b*)). This is a large complex of intracytoplasmic channels formed by the invagination of the plasma membrane forming multiple channels throughout the entire cytoplasm[16,19]. Their lumen remain connected to the extracellular space, as demonstrated by the penetration of electron dense tracers, like horseradish peroxidase. This membrane system expands very rapidly and becomes widespread within the whole cytoplasmic volume, expanding more than 700% within 72 hours. This system is designed to furnish a large reserve of membrane, which is destined to form the future platelets. The protein composition of both plasma membrane and demarcation membranes remains remarkably similar. The major platelet receptors, which are usual components of the mature MK membrane, are equally expressed within the demarcation membrane system: GpIIb/IIIa which becomes activatable only in the mature cell, the GPIb/IX/V complex, and GPIV (CD36) appear sequentially during early stages of maturation along with GPVI[20–23].

Secretion granules

The secretion granules of three main types are formed in parallel: alpha-granules, dense granules and lysosomes.

Cambridge Books Online © Cambridge University Press, 2009

Alpha-granules

These initially arise in the trans-Golgi network (TGN), originating from the assembly of endogenously synthesized proteins. They contain a wide variety of components. They are mostly involved in hemostasis such as the adhesive proteins (fibrinogen, von Willebrand factor, thrombospondin, fibronectin . . .) and other specific factors like β-thromboglobulin (β-tg) and platelet factor 4 (PF4); other components are implicated in wound healing, mainly growth factors (PDGF, TGF-β, EGF, VEGF). Alpha-granule components are not only synthesized by the MK itself but also originate from plasma proteins endocytosed during MK maturation[24–27]; endocytosis is either fluid-phase (albumin, immunoglobulins) or receptor-mediated, e.g. fibrinogen through its receptor GpIIb/IIIa[28,29]. Endocytosed proteins seem to appear later in the MK than endogenously synthesized proteins (von Willebrand Factor), and their distribution patterns within the maturing MK cytoplasm are distinct, i.e. centrifugal and maximal at the cell periphery for the former, but centripetal and predominant in the juxtanuclear area for the latter[30]. The alpha-granule membrane contains numerous receptors which appear during MK maturation, e.g. CD62p (P-selectin), GpIIb/IIIa, CD36, CD9, and PECAM1. Most of them appear to be expressed first at the plasma membrane, and become subsequently internalized. Tagging plasma membrane GpIIb/IIIa indicates that the internal pool of alpha granular GpIIb/IIIa directly originates from the internalized plasma membrane pool[31].

Dense granules

These are formed early during MK maturation, since specific components of their limiting membrane such as granulophysin, are detected in megakaryoblasts[32] but they acquire their dense content (Ca^{2+}, active uptake of serotonin) at the terminal stage of maturation. They also share some common components with the α-granular membrane, e.g. CD62p and GpIIb/IIIa[33,34]. An intermediary compartment, the multivesicular body has recently been identified as an apparent crossroad located downstream from TGN, where endogenously synthesized and endocytozed granule components are sorted to their correct respective intracellular locations[32,35].

Lysosomes

These are scarce and their content can be visualized by electron microscopic cytochemistry. They contain acid hydrolases, such as acid phosphatase, β-galactosidase and β-glucuronidase[36].

Mitochondria

Mitochondria are more sparse and smaller in size within the mature cell when compared to megakaryoblasts.

The endoplasmic reticulum

This is scarce and scattered in the cytoplasm. It contains the specific platelet peroxidase activity (PPO), which is also present in the perinuclear cisternae and reflects the cell content in cyclo-oxygenase[37].

Platelet production

Platelet production by MK still appears to be a poorly understood event. No consistent study has been able to document the phenomenon in vivo particularly within humans. Nevertheless, the mechanism of platelet formation and shedding by MK has been approached by several means. In the early 1970s, Thiery et al. observed preparations of bone marrow MKs squashed on a glass slide by using a microcinema technique. They could demonstrate the ability of MK to deform and extend long cytoplasmic filaments called proplatelets[38].

Mechanism and site of platelet release by MKs

Several theories have been raised concerning the formation of platelets by MKs: budding of platelets from the cell surface; delineation within the MK cytoplasm of small preformed cytoplasmic territories ready to fragment, eventually released at the end of cell maturation (static model)[39]; or demarcation membranes as a reservoir for the emission of long filaments named proplatelets which cross the endothelial barrier in order to enter the circulation, break, and form small fragments ready to detach as platelets (flow model)[40–45]. Recent images obtained on cells having reached full cytoplasmic maturity in culture, in the presence of TPO, favour a third hypothesis of proplatelet formation (Fig. 2.3). Although an extension of the above flow model, formation of platelets is apparently preceded by the extension of proplatelets as well as dilatation of the demarcation membrane system. The following events leading to platelet formation are sequentially observed in vitro[45]: peripheral redistribution and alignment of demarcation membranes; dilatation of the demarcation membranes; extension of proplatelets displaying regular constriction zones; with transverse microtubules disposed along longitudinal bundles of microtubules at the constriction zones; breakage at the constriction area and liberation of a newly formed platelet from the tip of the proplatelet where the classical loop of microtubules is formed[46] (Fig. 2.3, see colour plate, 2.4, 2.5(*a*)).

An alternative theory proposes that proplatelet fragmentation and platelet shedding may occur in the circulation,

Cambridge Books Online © Cambridge University Press, 2009

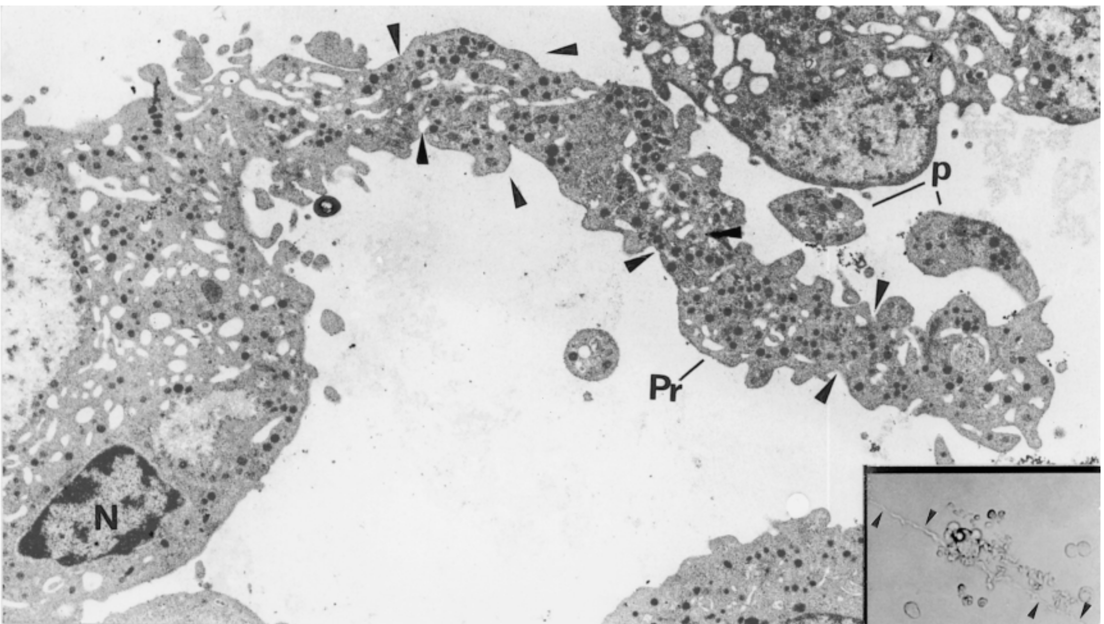


Fig. 2.3 (see also colour plate). After reaching full maturation, MK cytoplasm extends away from the cell core of the mother cell forming one or several long extensions called proplatelets (pr). Some platelets (p) have detached from its tip. N = nucleus (M × 3354).
Inset: Phase contrast microscopic view of a mature MK extending proplatelets (arrowheads).

following a circadian rhythm, with an apparent peak of platelet liberation in the early morning hours[47]. New platelets display longitudinal microtubules along their long axis, similar to the microtubule organisation in proplatelets[42,45] (Fig. 2.5(*b*)). Then they curve, both their extremities fuse, generating a central vacuole which tends to decrease until it vanishes, leading to the formation of a mature platelet with a circumferential marginal bundle of microtubules.

Transendothelial migration of MK: MK chemotaxis

MKs express the chemokine receptor CXCR4. The action of its ligand, the chemokine stromal cell-derived factor-1 (SDF-1) was found to induce MK chemotaxis, i.e. migration towards a chemotactic gradient[48]. The experiment was conducted in a modified Boyden chamber, showing that whole intact mature MKs can orientate themselves with the formation of a unilateral cytoplasmic pseudopod, the nuclear lobes being gathered at the opposite pole, and can migrate through a transwell of bone marrow endothelial cells. MKs are also able to react to agonists in a similar fashion to platelets[49] (Fig. 2.6), therefore their presence in the circulation might be pathogenic in some situations such as lung diseases. Thrombin induces dramatic morphological changes as early as the megakaryoblastic stage, showing that the protease-activated receptors (PAR) are expressed early on this cell line. Two other groups made comparable studies and also observed that CXCR4 expression increases with MK maturation, but mature MKs as well as platelets do not respond to SDF-1, suggesting that the signalling induced by SDF-1 is not operating: these results were interpreted as a potential mechanism for retaining immature MKs in the marrow[50,51].

Knockout and transgenic animal models

Targeted disruption of genes, i.e. the knockout approach has recently been extensively developed and has proved to provide useful models for understanding the role of receptors and transcription factors in MK differentiation. Knockout mice for either the mpl-receptor[52] or its major ligand TPO[53] develop severe thrombocytopenia, but platelet levels of approximately 10–15% of normal are still maintained. The transcription factor p45–NFE2 has also been shown to be essential for MK cytoplasmic development since NFE2 −/− mice fail to produce any platelets, although (dysmorphic) MKs are found within the bone marrow[54]. These cells remain with a cytoplasm of drastically reduced size and also fail to show any apparent signs of proplatelet formation[55]. In addition, the nucleus remains hypoploid. Thromboxane synthase and β1-tubulin are also absent

Cambridge Books Online © Cambridge University Press, 2009

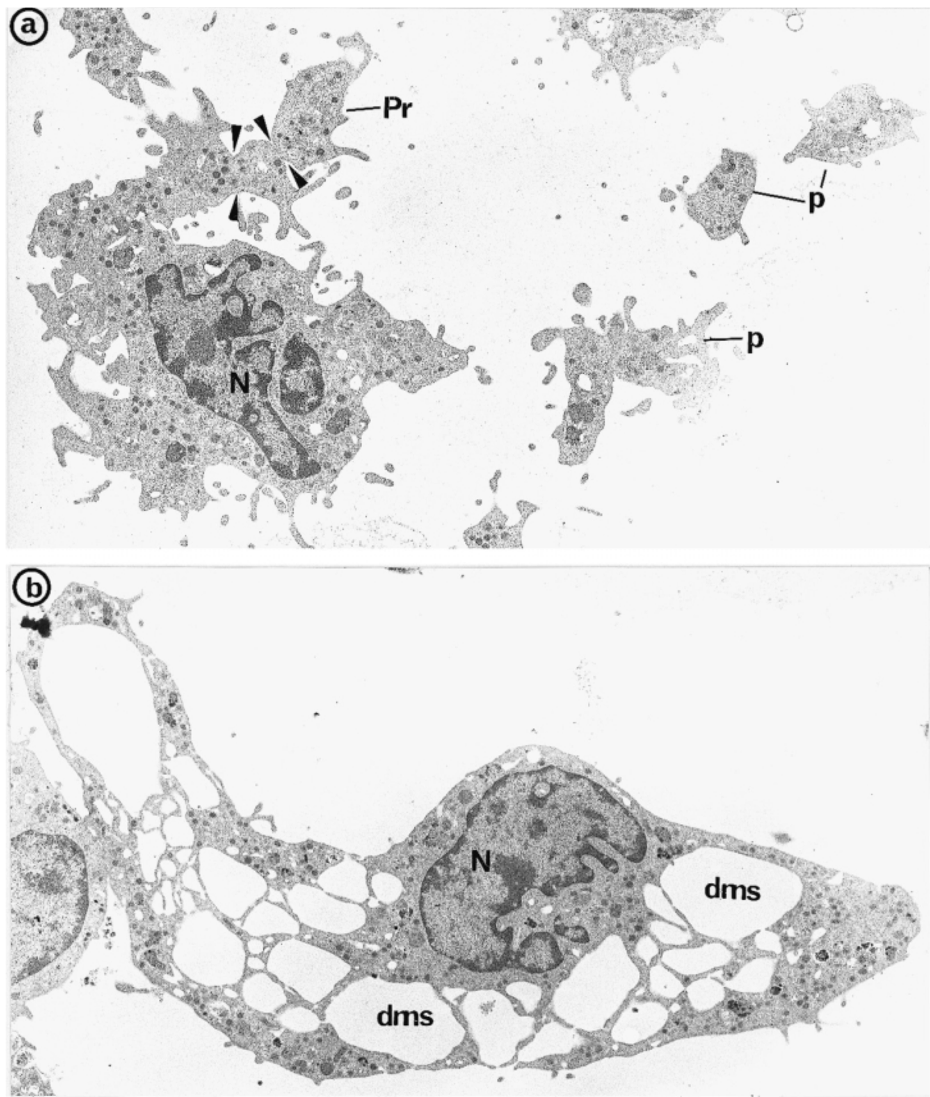


Fig. 2.4. At the platelet shedding stage. (*a*) The peripheral sheet of cytoplasm unfolds from the cell core, forming an elongated proplatelet process (pr). Constriction zones are regularly disposed along this cytoplasmic extension (arrowheads), delimitating future platelet territories. Platelets (p) detach from the tip of the proplatelet (M × 2660). (*b*) The MK demarcation membrane system (dms) widens and allows future proplatelets and platelet territories to be formed (arrowheads). N = nucleus (M × 2660)

from these MKs[56,57]. This is an important observation as β-tubulin is an important cytoskeletal protein within platelets and MK (endo)mitotic spindle structures. As a consequence of these defects, the thrombocytopenia induced in this model is more severe than for either c-mpl and TPO knockout mice. GATA1 knockout mice also exhibit severe thrombocytopenia with both a proliferation defect of MK progenitors and a marked cytoplasmic maturation defect leading to the production of giant platelets[58]. Mice lacking the transcription factor Fli1 (Fli1 −/− mice) develop thrombocytopenia with abnormal platelet morphology resembling the defects observed in Paris–Trousseau thrombocytopenia[59]. Finally, GpIb knockout mice present phenotype very close to the analagous human disease, Bernard–Soulier syndrome. The mice exhibit severe thrombocytopenia and giant platelets demonstrating the direct role of the receptor for MK cytoplasmic development and delineating platelet territories[60].

Cambridge Books Online © Cambridge University Press, 2009

Regulation of platelet production

At least three main successive events are involved in megakaryopoiesis. First, proliferation of progenitor cells (CD34+) evolving to more differentiated but less proliferative cells, expressing CD41, the thrombopoietin receptor (c-Mpl) and the alpha chemokine receptor CXCR4. Secondly, the megakaryoblastic stage where cells increase their DNA content without proliferating through the specific mechanism of endomitosis. Thirdly, the megakaryocytic stage, at which cells are involved in the maturation process that yields to proplatelet formation and platelet shedding (either *in situ* or after MK migration into the bone marrow capillary system and further fragmentation within the pulmonary blood circulation). Many cellular signals are required to regulate these stages, e.g. cytokine-induced proliferation and differentiation, cytokine-induced migration, cell–matrix interactions and cell–cell interactions (microenvironment). Megakaryopoiesis appears to be promoted by multiple interacting, often redundant mechanisms. We will now develop these mechanisms separately, especially the influence of growth factors, observations from both in vitro then in vivo experiments, preclinical studies and finally clinical trials and future prospects.

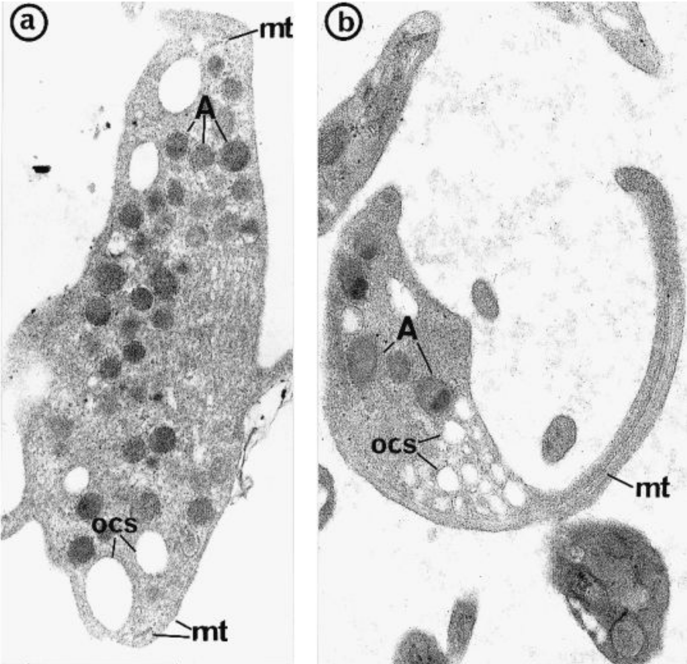


Fig. 2.5. (*a*) Ultrastructure of a platelet shed by a cultured MK in vitro. It resembles blood platelets, with an ellipsoid shape, peripheral microtubules (mt), scattered α-granules (A) and open canalicular system (ocs) (M×8220). (*b*) Ultrastructure of a platelet from the peripheral blood, with a spindle shape and a longitudinal bundle of microtubules (mt), which resembles the one found along proplatelets. This type of platelet may represent the newly born ones, encountered when platelet production is accelerated. A = α-granule (M×8220).

Thrombopoietin (TPO) search and discovery

It was suspected for a long time that a lineage specific growth factor, thrombopoietin (TPO), promotes megakaryopoiesis in a similar fashion to erythropoietin (EPO) in the model of erythropoiesis. This factor was eventually discovered from the study of the MPLV (myeloproliferative leukemia virus). This retrovirus, when acting on murine hematopoietic progenitors, induces myeloproliferative disorders, and MPLV-infected hematopoietic cells can grow and differentiate usually without a growth factor requirement[61]. From molecular studies, transforming properties of MPLV were attributed to v-mpl, a viral envelope-coding oncogene[62]. c-mpl, the human homologue of v-mpl, was then cloned, and the primary sequence suggested that the receptor was a member of the cytokine receptor superfamily[63,64]. c-mpl RNA appears to be specifically expressed within fetal liver cells, CD34+ cells, MK precursors, MKs and platelets. In mice, the c-mpl transcript is found in all hematopoietic tissues but thymic cells. In humans, c-mpl mRNA is not expressed in non-MK hematopoietic cells. Low expression has also been reported in endothelial cells. c-mpl mRNA expression was also found in almost all megakaryocytic-derived cell lines, e.g. UT-7, Mo7E, TF-1, HEL, DAMI, and KU-812[65]. c-mpl major expression is detected on primitive stem cells (CD34+/CD38−), CD34+, GpIIb/IIIa+ and on mature MKs and platelets[66]. Within hematopoietic progenitor cells in culture, transfected antisense oligodeoxynucleotides to c-Mpl mRNA totally inhibited the growth of MK colonies without inhibition of other lineage proliferation[67]. c-mpl-deficient mice also exhibited specific megakaryocytic lineage and platelet production defects[52]. These results therefore strongly implicated c-Mpl as a major MK-lineage specific growth factor receptor. Soon after the description of this specific receptor, a specific ligand and activator of c-Mpl was purified, sequenced and cloned using a number of different strategies by several research teams in 1994[68,55,69–73]. This ligand, named c-Mpl ligand or megapoietin, is now referred to as thrombopoietin (TPO).

Cambridge Books Online © Cambridge University Press, 2009

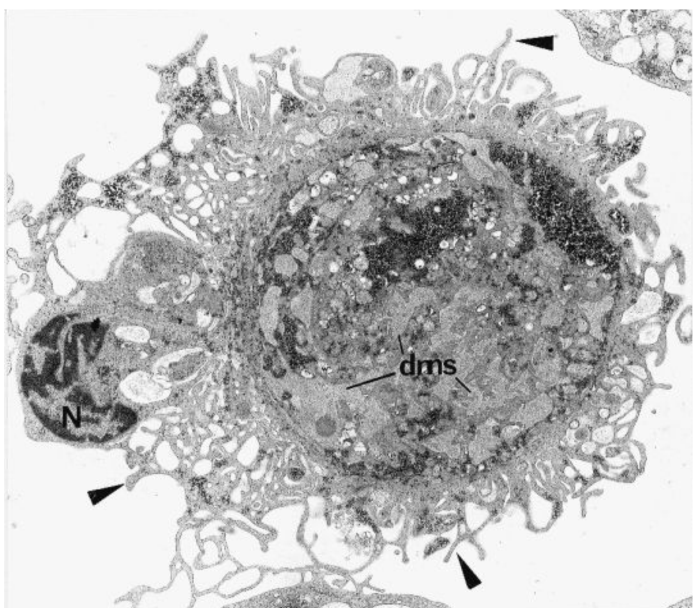


Fig. 2.6. Like platelets, MKs can become activated: thrombin induces shape change, pseudopod emission from the plasma membrane (arrowheads), organelle centralization and granule secretion into the dilated channels of the demarcation membrane system (dms). The nucleus (N) is pushed towards the cell periphery (M×2581).

TPO gene, cDNA and protein[68,74,75]

TPO cDNA (1.8 kb) reveals strong inter-species homologies (70 to 80%) and codes for a 353 amino acid protein. The TPO gene locus was reported on the long arm of the human chromosome 3 (3q26–27), a region previously shown to be involved in myeloproliferative disorders. TPO mRNA is expressed strongly in liver and kidney in humans. Expression could also be detected in smooth muscle cells and low amounts within bone marrow stromal and spleen cells; TPO mRNA can also be detected in primary cultures of endothelial cells.

TPO is a 75 to 80 kD protein, with two distinct domains: one N-terminal biologically active domain, 153 amino acids long, with 50% homology to EPO (EPO-like domain); and one carboxy-terminal domain, glycosylated, with no primary sequence homology to any other known cytokine. This latter domain has a putative role in protein secretion and in protein lifespan increase in the systemic circulation.

In cell culture media, in vivo, and in clinical trials, two recombinant forms of TPO have been used: one is the *Escherichia coli* expressed part of the EPO-like domain of TPO, the rHu-MGDF for recombinant human MK growth and development factor. A polyethylene glycol (PEG) derivative form is sometimes used, the PEG-rHuMGDF. It has a longer serum half-life. Another form is a full-length recombinant TPO glycosylated and produced in mammalian cells, named rhTPO. Moreover, and importantly, c-Mpl ligand peptides without any primary sequence homology to the natural c-Mpl ligand compete specifically with TPO for the binding of c-Mpl and display similar megakaryopoietic properties[76].

TPO production and regulation

TPO synthesis occurs mainly in liver production. A lower TPO production takes place in the bone marrow stromal cells (which may constitute an important *in situ* source) and also in splenic cells.

The regulation of TPO level and its correlation to platelet production is the centre of debate. In mice, TPO mRNA expression in induced thrombocytopenic or thrombocythemic states was reported to be unchanged[77]. Moreover, in TPO −/+ genetically modified mice (heterozygous), a 50% reduction in platelet count but with no TPO mRNA overexpression, was observed[78]. These data therefore suggest that mRNA expression of TPO is constitutive.

The systemic level of TPO is usually inversely correlated to platelet count[72]. For instance, in c-mpl−/− mice, a low platelet count in conjunction with low MK number but high TPO plasma level are observed[78]. Transfusion of normal platelets into these mice induces a rapid decrease in TPO level[79].

Platelet mass as well as total MK total mass are responsible for TPO level regulation: internalization and subsequent degradation of TPO/c-Mpl complex in platelets (and also within MKs) explains the constant regulation of TPO concentration via a post-transcriptional mechanism[80,81]. A similar post-transcriptional regulation of macrophage colony-stimulating factor (M-CSF) level, by monocytes, was previously demonstrated[82].

Patients with immune thrombocytopenic purpura (ITP) often present with thrombocytopenia, an elevated number of bone marrow MKs, and normal systemic TPO level[83]. Implication of the bone marrow microenvironment has also been emphasised: in ITP patients, a correlation was observed between TPO level in the bone marrow and TPO mRNA expression in bone marrow stromal cells, showing that regulation mainly occurs *in situ*[84]. In bone marrow, TPO is provided by stromal cells, and its mRNA expression modulated by platelet alpha-granular proteins. Dual effects of transforming growth factor-beta 1 (TGF-beta1), a cytokine present at a high concentration in platelets, were

Cambridge Books Online © Cambridge University Press, 2009

reported, i.e. enhancement or inhibition of TPO mRNA expression in stromal cells[85,86]. However, there seems to be a consensus that TGF-beta exerts an inhibitory effect on MK growth (see further discussion on 'Negative regulation of megakaryopoiesis').

Autocrine and paracrine mechanisms could be involved to modulate TPO production by bone marrow stromal cells, and also by MK *in situ* release of α-granules proteins. Many interacting mechanisms then participate to regulate TPO levels.

TPO biological activities in vitro (cell culture)

Primary culture studies have established several megakaryopoietic effects of TPO. It appears to be multifunctional by inducing progenitor cell proliferation, MK colony-stimulation and MK maturation.

TPO can promote MK colony formation

In serum-free cultures TPO induces proliferation of hematopoietic progenitor cells (CD34+, GpIIb/IIIa + or −) from human or murine bone marrow. However, the proliferative effect is relatively weak and TPO only promotes small MK colony formation. To induce the formation of larger colonies from CD34+ cells, the additive effects of cytokines such as Steel factor or interleukin (IL)-3 are also required[87]. Moreover, TPO appears to be dispensable at this stage and a combination of Steel factor, IL-3 and IL-6 can induce similar MK colony formation and MK production from CD34+ cells[87].

TPO promotes MK differentiation and maturation

Endomitosis, cytoplasmic maturation and proplatelet formation can be obtained from either blood or bone marrow CD34+ cells, in serum-free media supplemented with TPO alone[88]. These effects were reported to be independent of other cytokine production from MKs, stromal cells, or other cells[74]. Cytoplasmic maturation and proplatelet formation were observed at both optical and ultrastructural levels[18,45]. TPO was reported as the strongest cytokine to induce MK maturation. A combination of cytokines, e.g. IL-11 especially have been shown to also support in vitro maturation[89].

TPO effect on proplatelet and platelet formation is controversial

In vitro produced platelets are morphologically and functionally normal[44,45]. However, TPO has no direct effect on MK cytoplasmic fragmentation and platelet production. The application of TPO on mature MKs in culture only gives limited proplatelet formation. TPO appears to promote this last stage by inducing proper megakaryocytic cytoplasmic maturation, a critical step for efficient platelet production[90–92]. TPO could be retrieved from culture media when MKs reach end-of-maturation stages without interfering in proplatelet and platelet production.

TPO has also in vitro effects on platelets

Normal platelets express a small number of TPO receptors[93] and TPO ligation to its receptor can induce platelet homodimerization and cell signal transduction. This cell signal transduction involves downstream events including tyrosine phosphorylation of the intracellular Mpl-R domain, various Janus proteins (JAK2, Tyk2 and sh2) and signal transducers and activators of transcription (STAT3 and STAT5). TPO primes platelet activation: it changes platelet aggregation induced by shear stress or agonists, and also increases fibrinogen binding, platelet adhesion to collagen and CD62p expression[94]. However, and importantly, the TPO levels in these experiments are much higher than physiological levels.

TPO shows pleiotropic biological effects and can act on various cell types

After c-Mpl ligation, TPO induces c-Mpl intracytoplasmic chain phosphorylation and consecutive activation of the JAK/STAT cell signal transduction pathway[95].

On hu-AML (acute myeloid leukemia) cells in culture, TPO can induce proliferative effects in a fraction of the cell population; some cells even undergo megakaryocytic differentiation associated with the expression of a set of erythroid/megakaryocyte-specific transcription factors such as NF-E2, GATA-1 and GATA-2; but the precise cascade of molecules involved in cell signal transduction remains to be elucidated[92,96]. These transcription factors are also involved in MK differentiation[97–99,58].

TPO are also reported to have positive effects on other hematopoietic lineages by synergistically and/or additively cooperating with several hematopoietic growth factors, as EPO by enhancing erythroid lineage production[100] and on early hematopoietic precursors, in synergy with other cytokines[88,101–102].

In vivo TPO biological activities: genetically manipulated mice confirm the major megakaryocytopoietic effects of TPO

Mpl−/− deficient mice express approximately 10% residual MKs and platelets with normal ultrastructural morphology

Cambridge Books Online © Cambridge University Press, 2009

and granule content, with excessive but usually non-lethal bleeding. This demonstrates that TPO is the major growth factor which regulates platelet production[77]. The residual platelet production also demonstrates that other redundant mechanisms also control a limited part of megakaryopoiesis. No significant decrease in white and red blood cells could be measured, but reduced quantity of other lineage progenitor cells were reported, confirming that TPO is efficient at a very early stage of hematopoiesis[103].

Overexpression of TPO gene transfected in murine hematopoietic stem cells leads to MK lineage hypertrophy, and thrombocythemia[104].

Other thrombopoietic growth promoting cytokines[105,106]

Before the discovery of TPO, various studies reported the megakaryopoietic action of cytokines such as GM-CSF, IL-3, IL-11, SCF, EPO, and G-CSF. However, interest in these cytokines has waned since TPO emerged as the principal thrombopoietic regulator. Although IL-3 and GM-CSF have clear thrombopoietic properties, clinical studies showed that these effects were not useful in vivo. The same observation was made for G-CSF. However, the IL-6 cytokine family was potentially more interesting. This family shares a signal-transducing pathway through the gp130 receptor, and includes IL-6, IL-11, oncostatin M (OSM) and the leukemia inhibitory factor (LIF). IL-11 and IL-6 exhibit both pleiotropic and some common hematopoietic effects. Both however seem to be dispensable for megakaryopoiesis. Despite this, a synergy of IL-3, SCF and IL-11 with TPO on the MK colony-stimulating activity in culture, was reported: IL-11 alone or in synergy with IL-3 induces some patterns of megakaryocytic maturation (increased size, ploidy and acetylcholine-esterase production). In vivo animal experiments have also demonstrated that IL-11 can enhance both MK progenitor and platelet production. IL-11 was also used in clinical trials for the treatment of aplastic anemia.

Thrombopoietic negative regulators[107]

TGF-beta

TGF-beta especially isoform 1, is a growth inhibitor of megakaryocytopoiesis in vitro. It acts on human and murine MK precursors.

PF4 and other cytokines of the CXC family

Platelet factor 4, an alpha-granular platelet protein, inhibits in vitro proliferation of normal MK progenitors as well as progenitors from thrombocythemic patients[108]. PF4 has two different biologically active domains, C-terminal and overlapping C-terminal/central domains. The inhibitory activity could be reproduced with mimetic synthetic peptides. Interestingly it was abrogated by heparin, but only for the peptides mimicking the C-terminal sequence. PF4 inhibition of MK proliferation could, for instance, be reversed by glycoasminoglycans and heparinase[109–111]. PF4 exhibits a high affinity for heparin-like proteins, which bind growth factors as a prerequisite for their biological effects. One possible mechanism of PF4 action could then be mediated through the delocalization of other heparin-binding factors, i.e. IL-1, IL-3, Il-6 and GM-CSF.

Thrombin

This can inhibit MK growth through receptor-mediated activation. Thrombin also inhibits proplatelet formation from MKs[112] which is expected, knowing the drastic changes induced on MKs morphology and demarcation membrane distributions (Fig. 2.6).

Microenvironment and megakaryocytopoiesis regulation

Several lines of evidence have recently exemplified the implications of *in situ* mechanisms of megakaryopoiesis regulation:

Glycosaminoglycan components of the extracellular matrix interact with PF4 and other heparan sulfates bind the cytokines IL-1, Il-3, IL-6 and GM-CSF[111,113].

Gp Ib/IX expression on mature MKs could be a growth arrest signal of the lineage[114]; Bernard–Soulier, a bleeding disorder with giant platelets and thrombocytopenia accompanying the genetic deficiency of the GpIb/IX complex, demonstrates the link between actin, MK fragmentation and platelet formation.

Microvascular endothelial cells are known to be major contributors to hematopoietic cell regulation, by providing both cytokines and adhesion molecules. It was reported that the CXC chemokine SDF-1 could also be involved in the induction of cell proliferation of hematopoietic progenitors. This suggests cytokine induction that promotes early stage progenitor proliferation and late-stage platelet release that is not TPO dependent. This induces MK chemoattraction, adhesion to endothelial cells and transendothelial migration of MKs[48,115,116].

Preclinical studies

A major role for TPO in regulating platelet production has been confirmed by preclinical studies:

Normal mice which daily receive rhTPO during 7 days, develop a fourfold increase in platelet level after 5 days with a return to baseline within 10 days. Simultaneously, a

Cambridge Books Online © Cambridge University Press, 2009

20-fold increase in bone marrow MK progenitors and a tenfold increase in MK occur. These effects were much stronger than those obtained with any other cytokine combinations[69,72–73]. In myelosuppressed (either chemically or sublethally irradiated) animals[117], daily TPO injections can also reverse thrombocytopenia with a dose-dependent effect. Agonist peptides of the TPO receptor also increase platelet level of 80%, when injected into mice[75].

Perspectives of gene therapy from adenovirus delivering human TPO have also been tested in mice[118–121]: when normal mice are infected by TPO-adenovirus, the plasma level of TPO is high and platelet levels reach high levels. However, some mice developed antibodies, i.e. anti-murine TPO and subsequent thrombocytopenia, with depletion of bone marrow MKs and of bone marrow hematopoietic progenitors. This suggests that the transgene can induce immune dysfunction and auto-immune disorders. In mice with immune defects, nude mice, severe combined immune deficient (SCID) or non-obese diabetic (NOD)–SCID mice, thrombocytosis was accompanied with granulocytosis and increased hematopoietic bone marrow progenitors. SCID but not NOD–SCID mice developed subsequent osteomyelofibrosis and extramedullar hematopoiesis. NOD–SCID mice have functionally deficient monocytes and macrophages that are active cells in osteomyelofibrosis genesis. These data demonstrate that a chronic high level expression of TPO can be deleterious.

Non-human primates treated with recombinant TPO during several days exhibit increased platelets counts with a dose-dependent correlation[122]. Platelet counts are significantly increased after a 6 day delay, peak within 13 days and return to baseline progressively after treatment arrest. In the bone marrow, MK number, volume and ploidy are also increased. No significant changes in platelet morphology, lifespan and reactivity are observed.

Human clinical trials

Initial trials demonstrated that PEG-rHuMGDF or rhTPO administered before chemotherapy to patients with cancer efficiently increased platelet levels, with normal morphological and functional features, and no evidence of spontaneous in vivo activation[123–125]. No effects on white blood cell count nor hematocrit were reported.

Trials using repetitive administrations of PEG-rHuMGDF were reported to induce severe and persistent thrombocytopenia after the appearance of neutralizing antibodies which cross-react with endogenous TPO[126,129]. Therefore, clinical developments of this recombinant form were terminated by the pharmaceutical company in September 1998[128]. In contrast, in a recent study conducted in the United Kingdom[130], healthy human volunteers received a unique PEG-rHuMGDF bolus vs. placebo, and no immune thrombocytopenia was observed. The platelet levels doubled, with a peak at 12 days of injection, and returned to basal level within 28 days. Functional normal and viable platelets were produced with increased platelet lifespan and turnover. A 1.6 fold MK increase was observed in the bone marrow. Moreover, since TPO increases platelet aggregative reponsiveness to physiologic agonists in vitro, it was then anticipated that TPO treatment could induce vascular thrombotic secondary effects. But no deleterious thrombotic effects were reported in this study.

Therefore, rhTPO appears for the moment as the most specific, effective and safe growth factor for increasing platelet production. It is used for avoiding platelet transfusions and for accelerating thrombocytopenia recovery after chemotherapy of cancer patients. The presumed non-antigenic TPO mimetic peptides are under evaluation for their therapeutic interests[131], and seem to be less immunogenic than PEG-rHuMGDF, but still require extensive clinical investigation[132].

References

1 Lichtman MA, Chamberlain JK, Simon W, Santillo PA. Parasinusoidal location of megakaryocytes in marrow: a determinant of platelet release. *Am J Hematol* 1978; 4: 303–312.

2 Mohle R, Green D, Moore MA, Nachman RL, Rafii S. Constitutive production and thrombin-induced release of vascular endothelial growth factor by human megakaryocytes and platelets. *Proc Natl Acad Sci USA* 1997; 94: 663–668.

3 Almeida-Porada G, Ascensao JL. Isolation, characterization, and biologic features of bone marrow endothelial cells. *J Lab Clin Med* 1996;128:399–407.

4 Breton-Gorius J. On the alleged phagocytosis by megakaryocytes. *Br J Haematol* 1981; 47: 635.

5 Schmitt A., Wendling F, Guichard J, Drouin A, Cramer EM. Pathological interaction between megakaryocytes and PMN in idiopathic myelofibrosis. *Blood* 2000; 96: 1342–7.

6 Davis RE, Stenberg PE, Levin J, Beckstead JH. Localization of megakaryocytes in normal mice and following administration of platelet antiserum, 5-fluorouracil, or radiostrontium: evidence for the site of platelet production. *Exp Hematol* 1997; 25: 638–648.

7 Schmitt A, Cramer EM. *Of Mice and Men*; *Exp Hematol* in press.

Cambridge Books Online © Cambridge University Press, 2009

8 Tingaard-Pedersen N. Occurrence of megakaryocytes in various vessels and their retention in the pulmonary capillaries in man. *Scand J Haematol* 1978; 21: 369.
9 Tavassoli M, Aoki M. Migration of entire megakaryocytes through the marrow-blood barrier. *Br J Haematol* 1981; 48: 25–29.
10 Levine RF, Eldor A, Shoff PK, Kirwin S, Tenza D, Cramer EM. Circulating megakaryocytes: delivery of large numbers of intact, mature megakaryocytes to the lungs. *Eur J Haematol* 1993; 51: 233–246.
11 Vainchenker W, Bouguet J, Guichard J, Breton-Gorius J. Megakaryocyte colony formation from human bone marrow precursors. *Blood* 1979; 54: 940–945.
12 Breton-Gorius J, Vainchenker W. Expression of platelet proteins during the in vitro and in vivo differentiation of megakaryocytes and morphological aspects of their maturation. *Semin Hematol* 1986; 23: 43–67.
13 Tronik-Le Roux D, Roullot V, Schweitzer A, Berthier R, Marguerie G. Suppression of erythro-megakaryocytopoiesis and the induction of reversible thrombocytopenia in mice transgenic for the thymidine kinase gene targeted by the platelet glycoprotein alpha IIb promoter. *J Exp Med* 1995; 181: 2141–2151.
14 Breton Gorius J, Guichard J. Ultrastructural localization of peroxidase activity in human platelets and megakaryocytes. *Am J Pathol* 1972; 66: 227–293.
15 Williams N, Levine RF. The origin, development and regulation of megakaryocytes. *Br J Haematol* 1982; 52: 173–180.
16 Behnke O. An electron microscope study of megakaryocytes of rat bone marrow. I. The development of the demarcation membrane system and the platelet surface coat. *J Ultrastruct Res* 1968; 24: 412–433.
17 Nagata Y, Muro Y, Todokoro K. Thrombopoietin-induced polyploidization of bone marrow megakaryocytes is due to a unique regulatory mechanism in late mitosis. *J Cell Biol* 1997; 139: 449–457.
18 Vitrat N, Cohen-Solal K, Pique C et al. Endomitosis of human megakaryocytes are due to abortive mitosis. *Blood* 1998; 91: 3711–3723.
19 Cramer EM. Megakaryocyte structure and function. *Curr Opin Hematol* 1999; 6: 354–361.
20 Cramer EM, Savidge GF, Vainchenker W et al. Alpha-granule pool of glycoprotein IIb-IIIa in normal and pathologic platelets and megakaryocytes. *Blood* 1990; 75: 1220–1227.
21 Berlanga O, Bobe R, Becker M et al. Expression of the collagen receptor glycoprotein VI during megakaryocyte differentiation. *Blood* 2000: 2740–2745.
22 Debili NCI, MassÈ J-M, Guichard J, Katz A, Breton-Gorius J, Vainchenker W. Expression of CD34 and platelet glycoproteins during human megakaryocytic differentiation. *Blood* 1992; 80: 3022–3035.
23 Berger G, Caen J-P, Berndt MC, Cramer EM. Ultrastructural demonstration of CD36 in the alpha-granule membrane of human platelets and megakaryocytes. *Blood* 1993; 82: 3034–3044.
24 Harrison P, Savidge GF, Cramer EM. The origin and physiological relevance of alpha-granule adhesive proteins. *Br J Haematol* 1990; 74: 125–130.
25 Cramer EM, Debili N, Martin JF et al. Uncoordinated expression of fibrinogen compared with thrombospondin and von Willebrand factor in maturing human megakaryocytes. *Blood* 1989; 73: 1123–1129.
26 Harrison P, Wilbourn B, Debili N et al. Uptake of plasma fibrinogen into the alpha-granules of human megakaryocytes and platelets. *J Clin Invest* 1989; 84: 1320–1324.
27 Handagama P, Rappolee DA, Werb Z, Levin J, Bainton DF. Platelet alpha-granule fibrinogen, albumin, and immunoglobulin G are not synthesized by rat and mouse megakaryocytes. *J Clin Invest* 1990; 86: 1364–1368.
28 George JN. Platelet immunoglobulin G: its significance for the evaluation of thrombocytopenia and for understanding the origin of alpha-granule proteins. *Blood* 1990; 76: 859–870.
29 Handagama P, Bainton DF, Jacques Y, Conn MT, Lazarus RA, Shuman MA. Kistrin, an integrin antagonist, blocks endocytosis of fibrinogen into guinea pig megakaryocyte and platelet alpha-granules. *J Clin Invest* 1993; 91: 193–200.
30 De Larouzière V, Brouland JP, Souni F, Drouet L, Cramer EM. Inverse immunostaining pattern for synthesized versus endocytosed alpha-granule proteins in human bone marrow megakaryocytes. *Br J Haematol* 1998; 101: 618–625.
31 Masse JM, Perlemuter K, Debili N, Letestu R, Castaigne A, Cramer EM. Intracellular trafficking of the alphaIIbbeta3 receptor antagonist, abciximab, in normal and Glanzmann's disease megakaryocytes. *Br J Haematol* 1999; 107: 720–730.
32 Youssefian T, Cramer EM. Megakaryocyte dense granule components are sorted in multivesicular bodies. *Blood* 2000; 95: 4004–4007.
33 Israels SJ, Gerrard JM, Jacques YV et al. Platelet dense granule membranes contain both granulophysin and P-selectin (GMP-140). *Blood* 1992; 80: 143–152.
34 Youssefian T, Masse J-M, Rendu F, Guichard J, Cramer EM. Platelet dense granule membrane contains glycoproteins Ib and IIb–IIIa. *Blood*, 1997; 89: 4047–4057.
35 Heijnen HF, Debili N, Vainchencker W, Breton-Gorius J, Geuze HJ, Sixma JJ. Multivesicular bodies are an intermediate stage in the formation of platelet alpha-granules. *Blood* 1998; 91: 2313–2325.
36 Bentfeld-Barker ME, Bainton DF. Identification of primary lysosomes in human megakaryocytes and platelets. *Blood* 1982; 59: 472–481.
37 Gerrard JM, White JG, Rao GH, Townsend D. Localization of platelet prostaglandin production in the platelet dense tubular system. *Am J Pathol* 1976; 83: 283–294.
38 Thiery JP, Bessis M. Mécanisme de la plaquettogenèse. *Rev d'Hematol* 1965; 11: 162.
39 Trowbridge EA, Harley PJ. A computer model of the random binary sequential division of megakaryocyte cytoplasm to produce platelets. *Phys Med Biol* 1984; 29: 1477–1487.
40 Radley JM, Scurfield G. The mechanism of platelet release. *Blood* 1980; 56: 996.

Cambridge Books Online © Cambridge University Press, 2009

41 Leven RM, Yee MK. Megakaryocyte morphogenesis stimulated in vitro by whole old partially fractionated thrombocytopenic plasma: a model system for the study of platelet formation. *Blood* 1987; 69: 1046–1052.
42 Tablin F, Castro M, Leven RM. Blood platelet formation in vitro: the role of the cytoskeleton in megakaryocyte fragmentation. *J Cell Sci* 1990; 97: 59–70.
43 Topp KS, Tablin F, Levin J. Culture of isolated bovine megakaryocytes on reconstituted basement membrane matrix leads to proplatelet process formation. *Blood* 1990; 76: 912–924.
44 Choi ES, Nichol JL, Hokom MM, Hornkoh AC, Hunt P. Platelets generated in vitro from proplatelet-displaying human megakaryoyctes are functional. *Blood* 1995; 85: 402–413.
45 Cramer EM, Norol F, Guichard J et al. Ultrastructure of platelet formation by human megakaryocytes cultured with the Mpl ligand. *Blood* 1997; 89: 2336–2346.
46 Italiano JE Jr, Lecine P, Shivdasani RA, Hartwig JH. Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *J Cell Biol* 1999; 147(6): 1299–1312.
47 Behnke O, Forer A. From megakaryocytes to platelets: platelet morphogenesis takes place in the bloodstream. *Eur J Haematol* 1998; 60: 3–24.
48 Hamada T, Mohle R, Hesselgesser J, Hoxie J, Nachman RL et al. Transendothelial migration of megakaryoyctes in response to stromal cell-derived factor 1 (SDF-1) enhances platelet formation. *J Exp Med* 1998; 188: 539–548.
49 Cramer EM, Massé J-M, Caen J-P et al. Effect of thrombin on maturing human megakaryocytes. *Am J Pathol* 1993; 143: 1498–1508.
50 Kowalska MA, Ratajczak J, Hoxie J et al. Megakaryocyte precursors, megakaryocytes and platelets express the HIV coreceptor CXCR4 on their surface: determination of response to stromal-derived factor-1 by megakaryocytes and platelets. *Br J Haematol* 1999; 104: 220–229.
51 Riviere C, Subra F, Cohen-Solal K et al. Phenotypic and functional evidence for the expression of CXCR4 receptor during megakaryocytopoiesis. *Blood* 1999; 93: 1511–1523.
52 Gurney AL, Carver-Moore K, De Sauvage FJ, Moore MW. Thrombocytopenia in c-mpl-deficient mice. *Science* 1994; 265: 1445–1447.
53 de Sauvage FJ, Carver-Moore K, Luoh SM et al. Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. *J Exp Med* 1996; 183: 651–656.
54 Shivdasani RA, Rosenblatt MF, Zucker-Franklin D et al. Transcription factor NF-E2 is required for platelet formation independent of the actions of thrombopoietin/MGDF in megakaryocyte development. *Cell* 1995; 81: 695–704.
55 Lecine P, Villeval JL, Vyas P, Swencki B, Xu Y, Shivdasani RA. Mice lacking transcription factor NF-E2 provide in vivo validation of the proplatelet model of thrombocytopoiesis and show a platelet production defect that is intrinsic to megakaryocytes. *Blood* 1998; 92: 1608–1616.
56 Deveaux S, Cohen-Kaminsky S, Shivdasani RA et al. p45 NF-E2 regulates expression of thromboxane synthase in megakaryocytes. *EMBO J* 1997; 16: 5654–5661.
57 Lecine P, Italiano JE Jr, Kim SW, Villeval JL, Shivdasani RA. Hematopoietic-specific beta 1 tubulin participates in a pathway of platelet biogenesis dependent on the transcription factor NF-E2. *Blood* 2000; 96: 1366–1373.
58 Shivdasani R, Fujiwara Y, Mc Devit M, Orkin S. A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. *EMBO J* 1997; 16: 3965–3973.
59 Hart A, Melet F, Grossfeld P et al. Fli-1 is required for murine vascular and megakaryocytic development and is hemizygously deleted in patients with thrombocytopenia. *Immunity* 2000; 13: 167–177.
60 Ware J, Russell S, Ruggeri ZM. Generation and rescue of a murine model of platelet dysfunction: the Bernard–Soulier syndrome. *Proc Natl Acad Sci USA* 2000; 97: 2803–2808.
61 Wendling F, Varlet P, Charon M, Tambourin P. MPLV: a retrovirus complex inducing an acute myeloproliferative leukemic disorder in adult mice. *Virology* 1986; 149: 242–246.
62 Souyri M, Vigon I, Penciolelli JF, Heard JM, Tambourin P, Wendling F. A putative truncated cytokine receptor gene transduced by the myeloproliferative leukemia virus immortalizes hematopoietic progenitors. *Cell* 1990; 63: 1137–1147.
63 Vigon I, Mornon JP, Cocault L et al. Molecular cloning and characterization of MPL, the human homolog of the v-mpl oncogene: identification of a member of the hematopoietic growth factor receptor superfamily. *Proc Natl Acad Sci USA* 1992; 89: 5640–5644.
64 Skoda RC, Seldin DC, Chiang MK, Peichel CL, Vogt TF, Leder P. Murine c-mpl: a member of the hematopoietic growth factor receptor superfamily that transduces a proliferative signal. *EMBO J* 1993; 12: 2645–2653.
65 Vigon I, Florindo C, Fichelson S et al. Characterization of the murine Mpl proto-oncogene, a member of the hematopoietic cytokine receptor family: molecular cloning, chromosomal location and evidence for a function in cell growth. *Oncogene* 1993; 8: 2607–2615.
66 Debili N, Wendling F, Cosman D et al. The Mpl receptor is expressed in the megakaryocytic lineage from late progenitors to platelets. *Blood* 1995; 85: 391–401.
67 Methia N, Louache F, Vainchenker W, Wendling F. Oligodeoxynucleotides antisense to the proto-oncogene c-mpl specifically inhibit in vitro megakaryocytopoiesis. *Blood* 1993; 82: 1395–1401.
68 Bartley TD, Bogenberger J, Hunt P et al. Identification and cloning of a megakaryocyte growth and development factor that is a ligand for the cytokine receptor Mpl. *Cell* 1994; 77: 1117–1124.
69 Kaushansky K, Lok S, Holly RD et al. Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. *Nature* 1994; 369: 568–571.
70 Lok S, Kaushansky K, Holly RD et al. Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo. *Nature* 1994; 369: 565–568.

Cambridge Books Online © Cambridge University Press, 2009

71 Sohma Y, Akahori H, Seki N et al. Molecular cloning and chromosomal localization of the human thrombopoietin gene. *FEBS Lett* 1994; 353: 57–61.

72 Wendling F, Maraskovsky E, Debili N et al. cMpl ligand is a humoral regulator of megakaryocytopoiesis. *Nature* 1994; 369: 571–574.

73 Kuter DJ, Beeler DL, Rosenberg RD. The purification of megapoietin: a physiological regulator of megakaryocyte growth and platelet production. *Proc Natl Acad Sci USA* 1994; 91: 11104–11108.

74 Gurney AL, Kuang WJ, Xie MH, Malloy BE, Eaton DL, de Sauvage FJ. Genomic structure, chromosomal localization, and conserved alternative splice forms of thrombopoietin. *Blood* 1995; 85: 981–988.

75 Kaushansky K. Thrombopoietin: the primary regulator of platelet production. *Blood* 1995; 86: 419–431.

76 Cwirla SE, Balasubramanian P, Duffin DJ et al. Peptide agonist of the thrombopoietin receptor as potent as the natural cytokine. *Science* 1997; 276: 1696–1699.

77 Stoffel R, Wiestner A, Skoda RC. Thrombopoietin in thrombocytopenic mice: evidence against regulation at the mRNA level and for a direct regulatory role of platelets. *Blood* 1996; 87: 567–573.

78 Bunting S, Widmer R, Lipari T et al. Normal platelets and megakaryocytes are produced in vivo in the absence of thrombopoietin. *Blood* 1997; 90: 3423–3429.

79 Fielder PJ, Hass P, Nagel M et al. Human platelets as a model for the binding and degradation of thrombopoietin. *Blood* 1997; 89: 2782–2788.

80 Kuter DJ, Rosenberg RD. The reciprocal relationship of thrombopoietin (c-Mpl ligand) to changes in the platelet mass during busulfan-induced thrombocytopenia in the rabbit. *Blood* 1995; 85: 2720–2730.

81 Shivdasani RA, Fielder P, Keller GA, Orkin SH, de Sauvage FJ. Regulation of the serum concentration of thrombopoietin in thrombocytopenic NF-E2 knockout mice. *Blood* 1997; 90: 1821–1827.

82 Bartocci A, Mastrogiannis DS, Migliorati G, Stockert RJ, Wolkoff AW, Stanley ER. Macrophages specifically regulate the concentration of their own growth factor in the circulation. *Proc Natl Acad Sci USA* 1987; 84: 6179–6183.

83 Ichikawa N, Ishida F, Shimodaira S, Tahara T, Kato T, Kitano K. Regulation of serum thrombopoietin levels by platelets and megakaryocytes in patients with aplastic anaemia and idiopathic thrombocytopenic purpura. *Thromb Haemost* 1996; 76: 156–160.

84 Hirayama Y, Sakamaki S, Matsunaga T et al. Concentrations of thrombopoietin in bone marrow in normal subjects and in patients with idiopathic thrombocytopenic purpura, aplastic anemia, and essential thrombocythemia correlate with its mRNA expression of bone marrow stromal cells. *Blood* 1998; 92: 46–52.

85 Sungaran R, Chisholm OT, Markovic B, Khachigian LM, Tanaka Y, Chong BH. The role of platelet alpha-granular proteins in the regulation of thrombopoietin messenger RNA expression in human bone marrow stromal cells. *Blood* 2000; 95: 3094–3101.

86 Sakamaki S, Hirayama Y, Matsunaga T et al. Transforming growth factor-beta1 (TGF-beta1) induces thrombopoietin from bone marrow stromal cells, which stimulates the expression of TGF-beta receptor on megakaryocytes and, in turn, renders them susceptible to suppression by TGF-beta itself with high specificity. *Blood* 1999; 94: 1961–1970.

87 Debili N, Wendling F, Katz A et al. The Mpl-ligand or thrombopoietin or megakaryocyte growth and differentiative factor has both direct proliferative and differentiative activities on human megakaryocyte progenitors. *Blood* 1995; 86: 2516–2525.

88 Zeigler FC, de Sauvage F, Widmer HR et al. *In vitro* megakaryocytopoietic and thrombopoietic activity of c-mpl ligand (TPO) on purified murine hematopoietic stem cells. *Blood* 1994; 84: 4045–4052.

89 Broudy VC, Lin NL, Kaushansky K.Thrombopoietin (c-mpl ligand) acts synergistically with erythropoietin stem cell factor, and interleukin-11 to enhance murine megakaryocyte colony growth and increases megakaryocyte ploidy *in vitro*. *Blood* 1995; 85: 1719–1726.

90 Horie K, Miyazaki H, Hagiwara T et al. Action of thrombopoietin at the megakaryocyte progenitor level is critical for the subsequent proplatelet production. *Exp Hematol* 1997; 25: 169–176.

91 Cohen-Solal K, Debili N, Vainchenker W, Wendling F. Thrombopoietin (Mpl-ligand) and the regulation of platelet production. *Thromb Haemost* 1997; 78: 37–41.

92 Miyazaki H, Kato T. Thrombopoietin: biology and clinical potentials. *Int J Hematol* 1999; 70: 216–225.

93 Broudy VC, Lin NL, Sabath DF, Papayannopoulou T, Kaushansky K. Human platelets display high-affinity receptors for thrombopoietin. *Blood* 1997; 89: 1896–1904.

94 Oda A, Miyakawa Y, Druker BJ et al. Thrombopoietin-induced signal transduction and potentiation of platelet activation. *Thromb Haemost* 1999; 82: 377–384.

95 Gurney AL, Wong SC, Henzel WJ, de Sauvage FJ. Distinct regions of c-Mpl cytoplasmic domain are coupled to the JAK-STAT signal transduction pathway and Shc phosphorylation. *Proc Natl Acad Sci USA* 1995; 92: 5292–5296.

96 Matsumura I, Kanakura Y, Kato T et al. The biologic properties of recombinant human thrombopoietin in the proliferation and megakaryocytic differentiation of acute myeloblastic leukemia cells. *Blood* 1996; 88: 3074–3082.

97 Visvader JE, Elefanty AG, Strasser A, Adams JM. GATA-1 but not SCL induces megakaryocytic differentiation in an early myeloid line. *EMBO J* 1992; 11: 4557–4564.

98 Tsai FY, Keller G, Kuo FC et al. An early haemato-poietic defect in mice lacking the transcription factor GATA-2. *Nature* 1994; 371: 221–226.

99 Tsang AP, Visvader JE, Turner CA, Fujiwara Y, Yu C, Weiss MJ, Crossley M, Orkin SH. FOG, a multitype zinc finger protein, acts as a cofactor for transcription factor GATA-1 in erythroid and megakaryocytic differentiation. *Cell* 1997; 90: 109–119.

Cambridge Books Online © Cambridge University Press, 2009

100 Papayannopoulou T, Brice M, Farrer D, Kaushansky K. Insights into the cellular mechanisms of erythropoietin–thrombopoietin synergy. *Exp Hematol* 1996; 24: 660–669.
101 Ku H, Yonemura Y, Kaushansky K, Ogawa M. Thrombopoietin, the ligand for the Mpl receptor, synergizes with steel factor and other early acting cytokines in supporting proliferation of primitive hematopoietic progenitors of mice. *Blood* 1996; 87: 4544–4551.
102 Piacibello W, Sanavio F, Garetto L et al. Extensive amplification and self-renewal of human primitive hematopoietic stem cells from cord blood. *Blood* 1997; 89: 2644–2653.
103 Alexander WS, Roberts AW, Nicola NA, Li R, Metcalf D. Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl. *Blood* 1996; 87: 2162–2170.
104 Yan XQ, Lacey D, Hill D, Chen Y, Fletcher F, Hawley RG, McNiece IK. A model of myelofibrosis and osteosclerosis in mice induced by overexpressing thrombopoietin (mpl ligand): reversal of disease by bone marrow transplantation. *Blood* 1996; 88: 402–409.
105 Caen JP, Han ZC, Bellucci S, Alemany M. Regulation of megakaryocytopoiesis. *Haemostasis* 1999; 29: 27–40.
106 Begley CG, Basser RL. Biologic and structural differences of thrombopoietic growth factors. *Semin Hematol* 2000; 37: 19–27.
107 Han ZC, Lebeurier I, Caen JP. Negative regulation of megakaryocytopoiesis. In *The negative regulation of hematopoiesis*. Ed. M. Guigon Colloque INSERM John Libbey Eurotext Ldt. 1993; 229: 225–233.
108 Xi X, Caen JP, Fournier S et al. Direct and reversible inhibition of platelet factor 4 on megakaryocyte development from CD34+ cord blood cells: comparative studies with transforming growth factor beta1. *Br J Haematol* 1996; 93: 265–272.
109 Gewirtz AM, Calabretta B, Rucinski B, Niewiarowski S, Xu WY. Inhibition of human megakaryocytopoiesis *in vitro* by platelet factor 4 (PF4) and a synthetic COOH-terminal PF4 peptide. *J Clin Invest* 1989; 83: 1477–1486.
110 Gewirtz AM, Zhang J, Ratajczak J et al. Chemokine regulation of human megakaryocytopoiesis. *Blood* 1995; 86: 2559–2567.
111 Han ZC, Bellucci S, Shen ZX et al. Glycosaminoglycans enhance megakaryocytopoiesis by modifying the activities of hematopoietic growth regulators. *J Cell Physiol* 1996; 168: 97–104.
112 Hunt P, Hokom MM, Wiemann B, Leven RM, Arakawa T. Megakaryocyte proplatelet-like process formation in vitro is inhibited by serum prothrombin, a process which is blocked by matrix-bound glycosaminoglycans. *Exp Hematol* 1993; 21: 372–381.
113 Rider C. The selective binding of interleukines to glycosaminoglycans. *Leukemia* 1993; 7: 777–778.
114 Feng S, Christodoulides N, Kroll MH. The glycoprotein Ib/IX complex regulates cell proliferation. *Blood* 1999; 93: 4256–4263.
115 Wang JF, Park IW, Groopman JE. Stromal cell-derived factor-1 alpha stimulates tyrosine phosphorylation of multiple focal adhesion proteins and induces migration of hematopoietic progenitor cells: roles of phosphoinositide-3 kinase and protein kinase C. *Blood* 2000; 95: 2505–2513.
116 Lataillade JJ, Clay D, Dupuy C et al. Chemokine SDF-1 enhances circulating CD34(+) cell proliferation in synergy with cytokines: possible role in progenitor survival. *Blood* 2000; 95: 756–768.
117 Hokom MM, Lacey D, Kinstler OB et al. Pegylated megakaryocyte growth and development factor abrogates the lethal thrombocytopenia associated with carboplatin and irradiation in mice. *Blood* 1995; 86: 4486–4492.
118 Yan XQ, Lacey D, Hill D et al. A model of myelofibrosis and osteosclerosis in mice induced by overexpressing thrombopoietin (mpl ligand): reversal of disease by bone marrow transplantation. *Blood* 1996; 88: 402–409.
119 Abina MA, Tulliez M, Lacout C et al. Major effects of TPO delivered by a single injection of a recombinant adenovirus on prevention of septicemia and anemia associated with myelosuppression in mice: risk of sustained expression inducing myelofibrosis due to immunosuppression. *Gene Ther* 1998; 5: 497–506.
120 Villeval JL, Cohen-Solal K, Tulliez M et al. High thrombopoietin production by hematopoietic cells induces a fatal myeloproliferative syndrome in mice. *Blood* 1997; 90: 4369–4383.
121 Frey BM, Rafii S, Teterson M, Eaton D, Crystal RG, Moore MA. Adenovector-mediated expression of human thrombopoietin cDNA in immune-compromised mice: insights into the pathophysiology of osteomyelofibrosis. *J Immunol* 1998; 160: 691–699.
122 Farese AM, Hunt P, Grab LB, MacVittie TJ. Combined administration of recombinant human megakaryocyte growth and development factor and granulocyte colony-stimulating factor enhances multilineage hematopoietic reconstitution in nonhuman primates after radiation-induced marrow aplasia. *J Clin Invest* 1996; 97: 2145–2151.
123 Basser RL, Rasko JE, Clarke K et al. Thrombopoietic effects of pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF) in patients with advanced cancer. *Lancet* 1996; 348: 1279–1281.
124 Fanucchi M, Glaspy J, Crawford J et al. Effects of polyethylene glycol-conjugated recombinant human megakaryocyte growth and development factor on platelet counts after chemotherapy for lung cancer. *N Engl J Med* 1997; 336: 404–409.
125 O'Malley CJ, Rasko JE et al. Administration of pegylated recombinant human megakaryocyte growth and development factor to humans stimulates the production of functional platelets that show no evidence of in vivo activation. *Blood* 1996; 88: 3288–3298.
126 Kaushansky K. Thrombopoietin: from theory to reality. *Thromb Haemost* 1999; 82: 312–317.
127 Basser RL, Underhill C, Davis I et al. Enhancement of platelet recovery after myelosuppressive chemotherapy by recombinant human megakaryocyte growth and development factor

Cambridge Books Online © Cambridge University Press, 2009

in patients with advanced cancer. *J Clin Oncol* 2000; 18: 2852–2861.

128 Ozer H. Clinical issues and treatment advances in thrombocytopenia: introduction. *Semin Hematol* 2000; 37: 1–2.

129 Kuter DJ. Future directions with platelet growth factors. *Semin Hematol* 2000; 37: 41–49.

130 Harker LA, Roskos LK, Marzec UM et al. Effects of megakaryocyte growth and development factor on platelet production, platelet life span, and platelet function in healthy human volunteers. *Blood* 2000; 95: 2514–2522.

131 de Serres M, Ellis B, Dillberger JE et al. Immunogenicity of thrombopoietin mimetic peptide GW395058 in BALB/c mice and New Zealand white rabbits: evaluation of the potential for thrombopoietin neutralizing antibody production in man. *Stem Cells* 1999; 17: 203–209.

132 Demetri GD. Pharmacologic treatment options in patients with thrombocytopenia. *Semin Hematol* 2000; 37: 11–18.

Cambridge Books Online © Cambridge University Press, 2009

3

Morphology and ultrastructure of platelets

James G. White

University of Minnesota, Laboratory of Medicine and Pathology, Minneapolis, USA

Introduction

Platelets are deceptively simple cells. Their small size, lack of a nucleus and clear cytoplasm made recognition difficult for early microscopists[1–4]. As a result, platelets were the last of the cellular elements of circulating blood to be identified[5]. Also, recognition was not helped by the tendency of platelets to remain nondescript. They hide as far from the centre of the flowing column of blood as possible[6]. Other cells are busy carrying oxygen and removing carbon dioxide, supplying nutrients, transporting waste, reacting to foreign invaders or leaving the circulation to participate in inflammatory and immunological responses, but not platelets. This cell desires anonymity and remains as quiet as possible as it rolls along the intact endothelium for its 10 to 12 day lifespan. If it can retire to the spleen without becoming involved in any physiologic activity, the platelet's life can be considered a complete success.

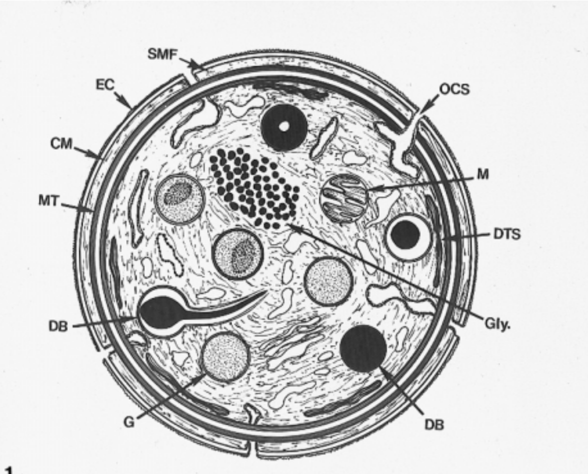
Thus, in this sense the platelet has no function in the circulation, except one: to be there when it is needed to keep blood flowing. It is the sentinel on guard at all times to react immediately at sites of vascular injury as soon as subendothelium is exposed. Within microseconds, platelets undergo dramatic changes in their morphology and biochemistry, fill the site of damage with aggregates to form a hemostatic plug, contract to prevent further loss of blood and restore integrity to the vascular system[7–10]. The platelet serves its function as sentinel of the circulation very well, but it does have a blind side. It often fails to distinguish its role in hemostasis from involvement in thrombosis. As a result, platelet participation in in vivo-occlusive phenomena leading to heart attacks, strokes or other ischemic events often overshadows its major contribution as the basic cellular unit of hemostasis[11].

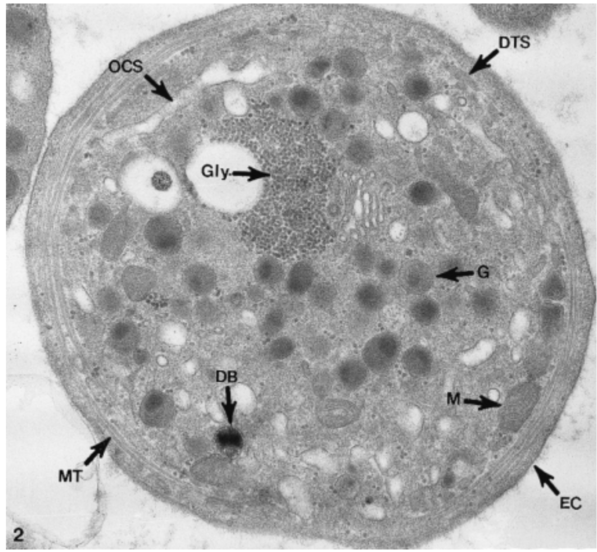
It is the task of the clinical and basic scientist to separate these two missions of the platelet, one desirable and the other potentially catastrophic, so that hemostasis can be achieved and thrombosis prevented. To accomplish this, we must learn as much about the platelet as possible. Our particular niche in this effort has been to dissect the fundamental relationships between platelet structure and function, and relate them to physiology and pathology. This chapter will focus on our current knowledge of platelet morphology and ultrastructure.

Structural organization

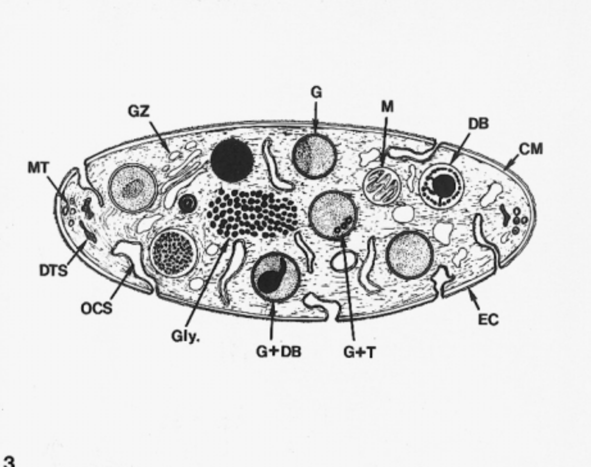
In order to simplify the complicated structural features of platelets and relate them to functional and biochemical activities, we divided the anatomy into three major regions[12,13] (Figs. 3.1–3.4). The peripheral zone consists of the external and internal membrane systems that provide the exposed surface of the platelet and walls of the tortuous channels making up the surface-connected open canalicular system (OCS). An exterior coat or glycocalyx, rich in glycoproteins, constitutes the outermost covering of the peripheral zone. Its chemical constituents provide the receptors for stimuli triggering platelet activation and the substrates for adhesion–aggregation reactions[14,15]. The middle layer of the peripheral zone is a typical unit membrane. It is rich in asymmetrically distributed phospholipids that provide an essential surface for interaction with coagulant proteins[16]. The area lying just inside the unit membrane represents the third component of the peripheral zone. It is closely linked to the unit membrane and translates signals received on the outside surface into chemical messages and physical alterations required for platelet activation[17].

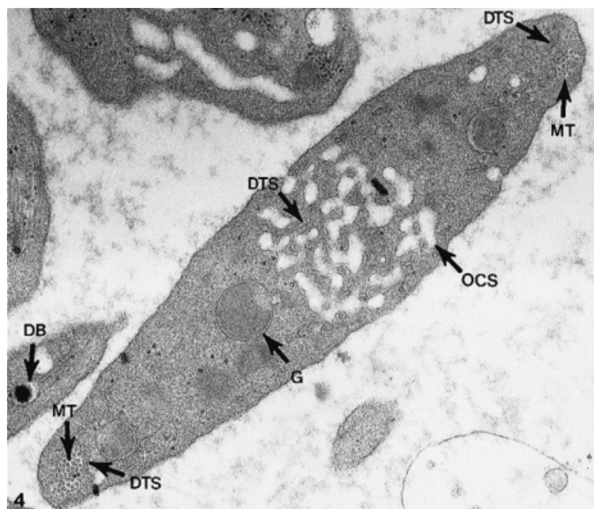
The internal membrane systems include the OCS, even though it is continuous with, and part of, the external membrane system. Channels of the dense tubular system





Figs. 3.1 and 3.2. Discoid platelets. The diagram in Fig. 3.1 summarizes ultrastructural features observed in thin sections of discoid platelets cut in the equatorial plane. Components of the peripheral zone include the exterior coat (EC), trilaminar unit membrane (CM), and submembrane area containing specialized filaments (SMF), which form the wall of the platelet and line channels of the surface-connected canalicular system (CS). The matrix of the platelet interior is the sol–gel zone containing actin microfilaments, the circumferential band of microtubules (MT), and glycogen (Gly). Formed elements embedded in the sol-gel zone include mitochondria (M), granules (G), and dense bodies (DB). Collectively they constitute the organelle zone. The membrane systems include the surface-connected canalicular system (CS) and the dense tubular system (DTS), which serve as the platelet sarcoplasmic reticulum. Fig. 3.2 is a platelet sectioned in the equatorial plane which reveals most of the structures indicated on the diagram (2 Mag ×28 000).





Figs. 3.3 and 3.4. Discoid platelets. The diagram in Fig. 3.3 summarizes the structures observed in platelets in cross-section, and Fig. 3.4 demonstrates an example of a cross-sectioned platelet. The designations for the structural features are given in the legend of Fig. 3.1. Occasionally, a Golgi Zone (GZ) can be found in circulating platelets; and some α-granules contain tubules (G.T.) resembling microtubules (4 Mag ×33 000).

(DTS) and the membrane complexes (MC) formed by elements of the OCS and DTS are internal membrane systems[18], but function with and are considered part of the peripheral zone.

The sol–gel zone is the matrix of the platelet cytoplasm. It contains several fibre systems in various states of polymerization that support the discoid shape of unaltered platelets and provide a contractile system involved in shape change, pseudopod extension, internal contraction, and secretion[19]. Elements of the contractile system appear to be major com-

Cambridge Books Online © Cambridge University Press, 2009

ponents, since they constitute approximately 30–50% of the total platelet protein. Masses as well as discrete particles of glycogen are distributed in the sol–gel matrix.

The organelle zone consists of granules, electron-dense bodies, peroxisomes, lysosomes, glycosomes, and mitochondria randomly dispersed in the cytoplasm. It serves in metabolic processes and for the storage of enzymes, non-metabolic adenine nucleotides, serotonin, a variety of protein constituents, and calcium destined for secretion.

Division of platelet anatomy into three functional zones is arbitrary. However, it does provide a framework for relating structure to function, as well as pathology.

Peripheral zone

It has long been appreciated that the platelet has a sphere of influence that extends beyond its physical limits[20,21]. The cell is affected by chemical signals and various phenomena occurring in flowing blood and in damaged areas of the microcirculation distant from its immediate environment. Chemical messengers originating from activated platelets influence other cells, including neighbouring platelets, leukocytes, and tissues of the vessel wall. Platelets are surrounded by dissolved constituents of blood, in particular the coagulant proteins, with which they interact. This relationship was recognized many years ago, and the term 'atmosphere plasmatique' was employed to describe it[20]. It is important to appreciate the dynamic implications of the peripheral zone and realize that the physical boundaries do not restrict or confine the platelet's biologic activities.

The major structural elements of the platelet peripheral zone are the cell surface and channels of the surface-connected OCS (Figs. 3.1–3.4). Since channels of the OCS represent invaginations of the cell wall, the peripheral zone is a continuous structure and the surface area exposed to surrounding plasma is far greater than might be expected from the usual measurements of the cell size or volume. In this sense, the platelet resembles a sponge more than any other blood cell[21]. The OCS shares the same characteristics of structural organization as more exposed portions of the cell membrane.

The peripheral zone is made up of the three structural domains: the exterior coat, the unit membrane, and the submembrane region as mentioned above. Most cell surfaces follow this same pattern of organization. However, differences in the chemical composition of each layer contribute to the unique properties of different cell types.

Exterior coat

The glycocalyx of the platelet is thicker and more concentrated than similar layers covering the outer surfaces of other blood cells (Fig. 3.5). Many different glycoproteins have been defined by immunocytochemistry and other methods on the exterior coat, including glycoproteins (GP), Ia, Ib, Ic, IIa, IIb, IIIa, IV, V, and VI[22]. Unlike other granule-containing cells, the platelet has a characteristic appearance, rather than the spherical form manifested by lymphocytes, monocytes, neutrophils and other organelle-filled leukocytes[12,13]. Its form is similar to that of the red blood cell, but platelets lack the biconcavity of the erythrocyte (Fig. 3.6). Thus, the platelet is the only flattened or slightly oval, disc-shaped cell in circulating blood. Yet, the smooth discoid appearance of the resting platelet is somewhat deceiving. Studies with electron dense tracers revealed that the exterior surface was frequently interrupted by pores at sites where channels of the open canalicular system (OCS) communicate with the outer membrane[23]. Freeze-fracture revealed pores of the OCS to particular advantage[24] (Fig. 3.7).

Despite the slight irregularity caused by communications of the OCS, techniques of light microscopy, particularly phase contrast and interference phase contrast, transmission electron microscopy of thin sections, whole mounts or replicas of freeze–fractured platelets and conventional scanning electron microscopy have all suggested

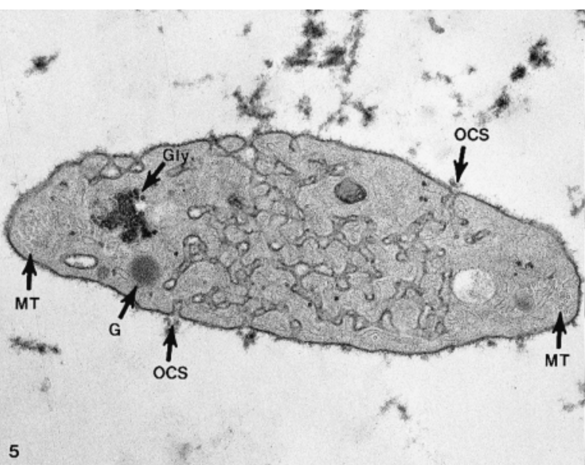


Fig. 3.5. Discoid platelet; the channel system. Cross-section of discoid platelet from sample of C-PRP stained with tannic acid during glutaraldehyde-osmium fixation. Circular profiles of microtubules (MT) supporting platelet discoid shape are evident at each pole of the cell. Tannic acid has acted as a mordant to deposit osmic acid in the exterior coat covering the exposed surface and channels of the open canalicular system (OCS). The OCS forms a system of interconnected channels stretching from one side of the cell to the other. Granules (G) and masses or single particles of glycogen (Gly) are dispersed in the cytoplasm (Mag × 33 000).

Cambridge Books Online © Cambridge University Press, 2009

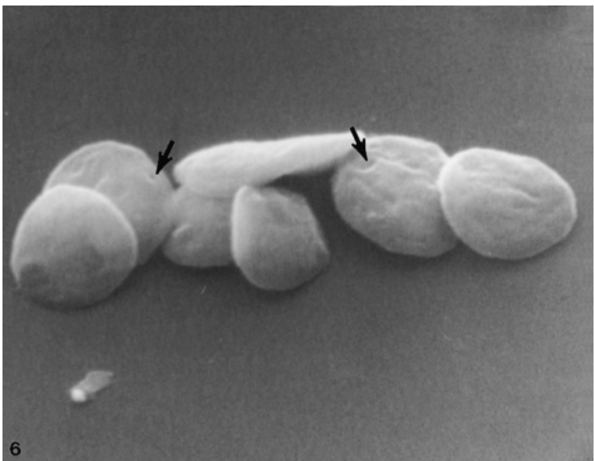


Fig. 3.6. Discoid platelets. The characteristic discoid form of the circulating blood platelet is evident in this scanning electron micrograph (SEM). Surfaces of the unaltered cells are relatively smooth, except for indentations (*arrows*) where channels of the surface-connected OCS communicate with the cell surface (Mag ×12 000).

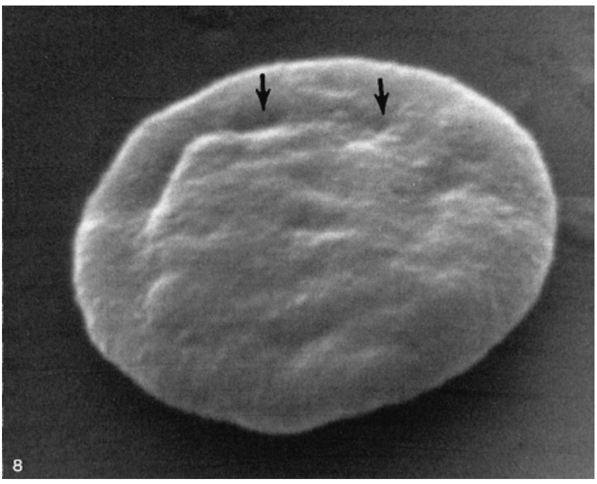


Fig. 3.8. Discoid platelet. Surface features of resting platelets are revealed to advantage in the SEM. The resting platelet has a discoid appearance and relatively smooth contours. Indentations (↑) represent communications between channels of the open canalicular system and the cell wall (Mag ×30 000).

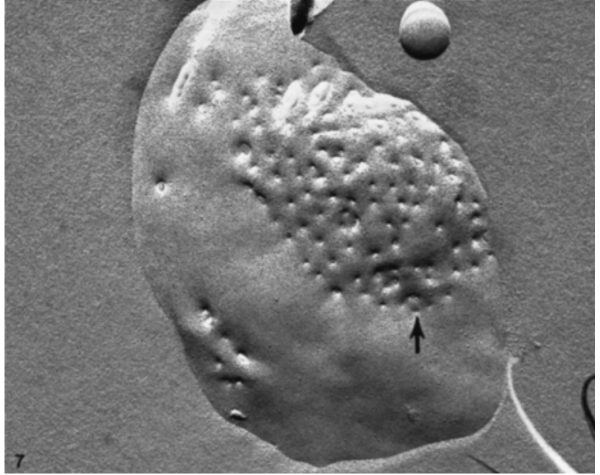


Fig. 3.7. Discoid platelet. Replicas of freeze–fractured platelets reveal communications between channels of the surface membrane to advantage. The fracture plane in this example has cleaved the lipid bilayer exposing the outside portion. Numerous pores (↑) are clustered in one area, and others are apparent along the outer edge (Mag ×30 000).

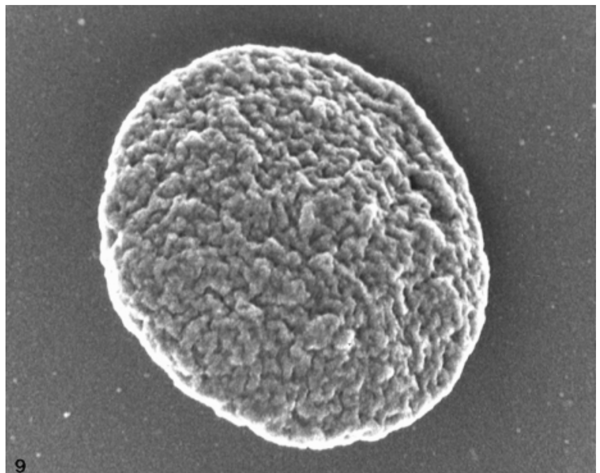


Fig. 3.9. Discoid platelet photographed in the low-voltage, high-resolution scanning electron microscope (LVHR-SEM). The outside of the cell resembles the surface of the brain. Gyri and sulci alternate in a convoluted fashion resulting in the wrinkled appearance (Mag ×40 000).

that the platelet surface is smooth[25] (Fig. 3.8). Low voltage high resolution scanning electron microscopy (LVHR-SEM), however, has suggested a different appearance[26]. Discoid platelets visualized by this technology have a corrugated appearance resembling the surface of the brain (Fig. 3.9). The gyri and sulci produced by the convolutions might result from shrinkage during preparation for study. However, red blood cells present in the same samples are smooth while leukocytes have a totally different appearance.

There are technical reasons why the appearance may have been missed by other methods. Light microscopy and its variants do not have the resolving power to identify the corrugations. Transmission electron microscopy sees

Cambridge Books Online © Cambridge University Press, 2009

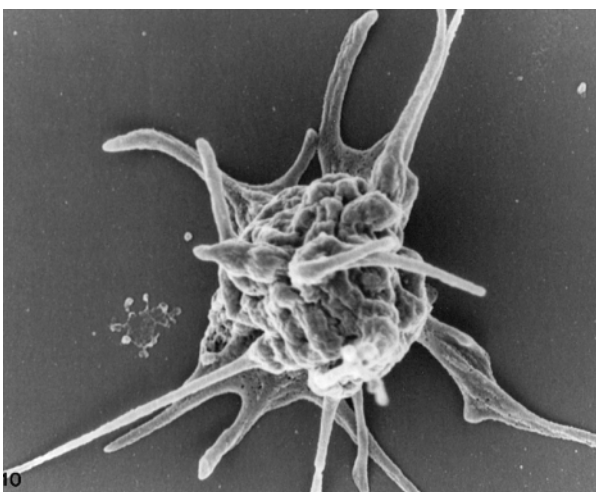


Fig. 3.10. Early dendritic platelet viewed by LVHR–SEM. Fine processes extend in all directions. Surfaces of the pseudopods are smooth compared to the central body from which they extend. Convolutions similar to those on the discoid cell in Fig. 3.8 are present on the platelet body (Mag ×16 000).

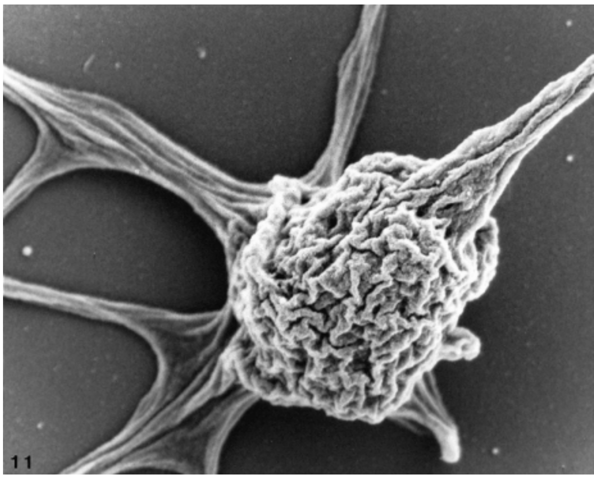


Fig. 3.11. Dendritic platelet. Pseudopods on this example are thicker than those on the cell in the last illustration. Convolutions on the central body extend into folds on the pseudopods (Mag × 33 000).

through transparent membranes of whole mounted platelets and surface details are not apparent. Freeze–fracture splits the membrane lipid bilayer and replicas do not reveal the outside returning from deeper in the specimen. The image generated tends to blur convolutions on the cell surface. Back-scattered electrons under conditions of low voltage derive mainly from the specimen surface and result, not only in high resolution, but a clearer definition of surface details. Thus, the convoluted surfaces on discoid platelets observed by LVHR–SEM may more accurately reflect the true organization of the resting platelet membrane than the smooth surfaced appearance provided by other techniques.

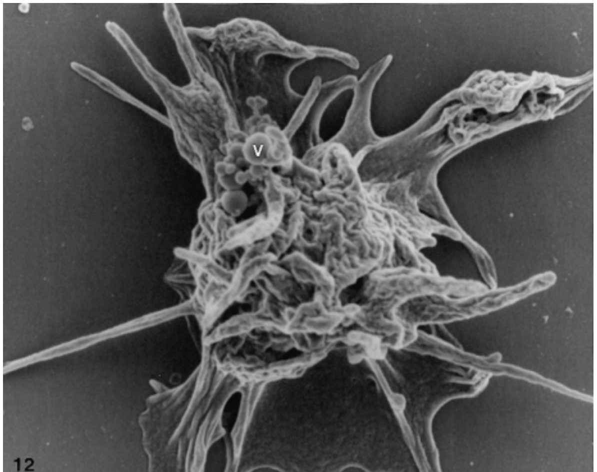


Fig. 3.12. Dendritic platelet. The complex process of surface interaction is revealed on this example. Some pseudopods are beginning to spread out on the formvar surface, while others remain long and smooth. Vesicular elements (↑) present on the convoluted central body may be microvesicles in the process of formation (Mag ×18 000).

Effects of surface activation

The advantage afforded by the convoluted surface of the resting platelet is readily apparent during interaction with surfaces. Extension of long, filiform processes is the first response of the platelet to the foreign surface (Fig. 3.10). They are not as delicate in appearance as those extending from other cell types, and are thicker and more spike-like on human platelets than on bovine cells. The difference is not due to the presence or absence of microtubules. All bovine platelet pseudopods contain at least one microtubule[27] while only one-third of the extensions emanating from human cells reveal a microtubule on cross-section.

The organization of the membrane covering the surface pseudopods on dendritic platelets viewed by LVHR–SEM is quite different than the irregular corrugations formed by gyri and sulci on discoid cells. Convolutions on the central body of the dendritic cell straighten out into long folds (Figs. 3.11, 3.12). The change in organization of surface membrane on extensions of the activated cell while the body retains the convoluted, brain-like pattern adds additional support to the suggestion that this appearance of the platelet surface is not an artefact of fixation. On late dendritic and early spread platelets the folds disappear resulting in a smooth membrane surface. (Fig. 3.13). Clearly, the

Cambridge Books Online © Cambridge University Press, 2009

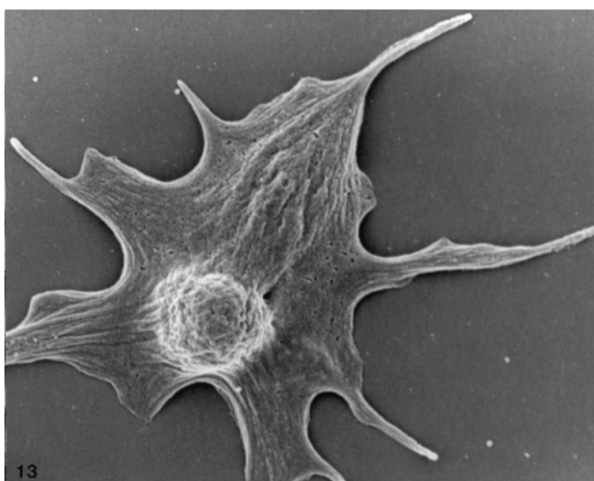


Fig. 3.13. Early spread platelet. The central body of the cell remains convoluted, but is gradually disappearing as the cytoplasm spreads and fills the spaces between pseudopods (Mag × 16 000).

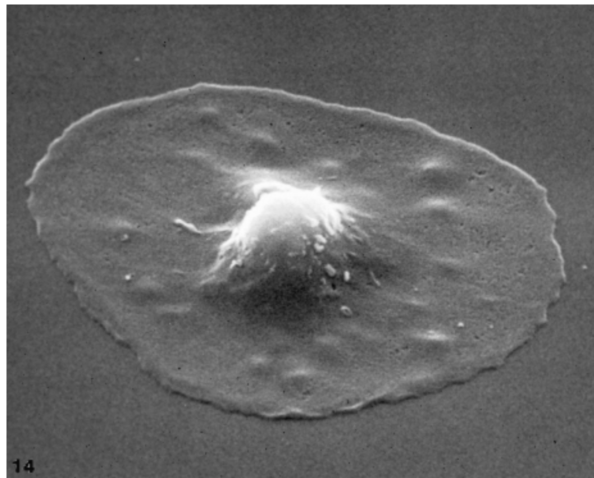


Fig. 3.14. Spread platelet viewed by conventional SEM (Mag × 12 000).

wrinkled surface of the resting cell provides a significant amount of membrane to cover the elongations as discoid cells are converted to dendritic forms.

A major objective of the platelet in hemostasis is to cover as much of the damaged area as possible to restore vascular integrity[11]. It accomplishes this mission by tethering, then sinking into the surface and expanding its cytoplasm to fill spaces between pseudopods. The process converts the flattened disc into a dendritic platelet, and then into a fully spread form. Convoluted membranes of the discoid cells play a significant role in the cytoplasmic expansion, as well as in pseudopod development. Gyri and sulci flatten into a smooth surface as the cytoplasm thins out and expands causing obliteration of the pseudopods. The result is a smooth-surfaced, completely spread cell resembling a pancake (Fig. 3.14).

The force generating mechanism underlying pseudopod formation and spreading is the assembly of cytoplasmic actin[28] (Fig. 3.15). Organization of the newly assembled filaments into parallel bundles produces battering rams to drive out spiky surface extensions. The convoluted surface would provide much less resistance to their extension than a skin tight, smooth membrane. Actin filaments assembling near the cell wall form a peripheral weave under the membrane forcing the expansion and filling in of spaces between pseudopods. Evidence favouring the proposed mechanism of platelet spreading has come from investigations employing cytochalasins B, D, and E[29]. Cytochalasins at concentrations that block development of new actin filaments prevent platelet pseudopod formation and spreading.

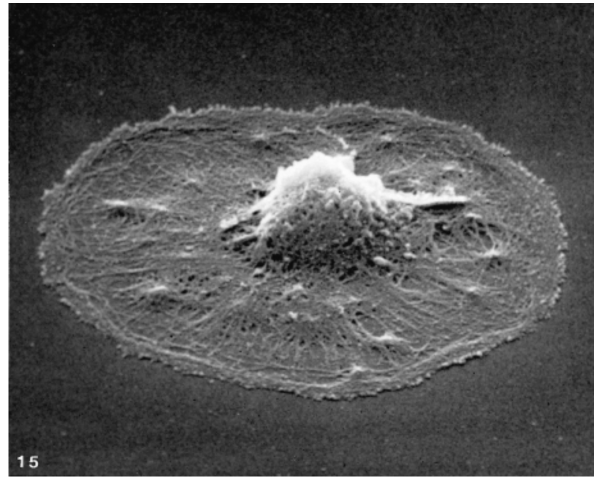


Fig. 3.15. Spread platelet extracted with Triton X-100 and examined in a conventional SEM. The cytoplasm is filled with detergent resistant actin filaments. Small raised areas in the cytoplasm of the extracted cell are adhesion plaques (Mag × 12 000).

During the conversion of discoid platelets to fully spread forms, the exposed surface area increases by over 400%. The origin of the membrane necessary for this large expansion requires consideration. Early workers may not have realized that the lipid bilayer of the surface membrane is incompressible like that of the red blood cell[30], and cannot expand in one direction without contracting in the other. The platelet has no capacity to synthesize lipids or protein. As a result, the expanded surface area cannot originate from new membrane synthesis. Studies in our laboratory

Cambridge Books Online © Cambridge University Press, 2009

suggested that channels of the surface-connected, open canalicular system might serve as a major membrane reservoir[31]. Experiments demonstrating that OCS channels are evaginated onto the cell surface during spreading have been reported[32], and their membranes clearly contribute to the increase in surface area.

However, the reservoir provided by the OCS may be too small to supply enough extra membrane for the fourfold increase in surface area observed during disc to spread platelet transformation. As a result, the corrugated appearance of the discoid platelet resembling the surface of the brain may be particularly important. It could, and apparently does, serve as an additional membrane reservoir for expansion of the platelet surface membrane. Discoid bovine platelets have the same crenelated appearance as human platelets, but lack channels of the open canalicular system[33]. Surface activation of bovine platelets results in extension of pseudopods and conversion into late dendritic forms, but they do not spread like human cells[34]. Yet, they do double their exposed surface area during disc to late dendritic transformation. The gyri and sulci on discoid bovine platelets appear to serve as the major, if not exclusive, source of additional membrane for this expansion.

Thus, the convoluted organization of resting platelets appears to serve a very useful purpose in the hemostatic reaction. It may also be involved in platelet pathology. Sims and his colleagues have shown that the complement proteins, C5b–9, cause release of membrane vesicles from the platelet surface enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity[35]. Similar microvesicles form on platelets during long-term storage and may represent a significant storage lesion (Fig. 3.12). The microvesicles clearly evolve from platelets, but the membrane of origin has not been clear until recently[36]. Platelets do not appear to decrease in volume despite considerable microvesicle formation, and internal membranes are an unlikely source because the cells remain intact. The pinching off of small vesicles from convoluted surfaces of platelets and membranes enclosing secretory organelles would seem a likely explanation for membrane loss without shrinkage of cell volume.

Platelet receptors: In order to exercise their fundamental role in hemostasis, platelets must adhere to the sites of damage on injured vessel walls. Discoid platelets in circulating blood or in platelet-rich plasma and washed suspensions in vitro are not sticky. Contact with subendothelium or other foreign surfaces results in adhesion and initiation of the shape change events. A variety of receptors covering the platelet membrane surface facilitate conversion of the cell to a sticky state[14–16,37–39]. Attachment to extracellular matrix proteins is mediated primarily by a family of heterodimeric cell surface proteins, each composed of α and β subunits. $\alpha_5\beta_1$ is a receptor for fibronectin and $\alpha_6\beta_1$ is a receptor for laminin on human platelets. Other platelet integrin receptors appear to be less specific. $\alpha_v\beta_3$ is a receptor for vitronectin, fibrinogen, fibronectin, thrombospondin and von Willebrand factor.

In addition, platelets carry non-integrin receptors on their surface, such as glycoprotein Ib–IX, the receptor for von Willebrand factor, and glycoprotein IV that binds thrombospondin and collagen. Glycoprotein Ia–IIa ($\alpha_2\beta_1$) is also a receptor for collagen as in GPVI. Although existence of so many molecules serving similar functions may seem redundant, they all appear to play a role in hemostasis.

The major platelet integrin is $\alpha_{IIB}\beta_3$ (glycoprotein IIb–IIIa). About 80 000 copies of $\alpha_{IIB}\beta_3$ are detectable on resting platelets. The complex is not expressed until the cell is activated in suspension or by surfaces. As soon as activation occurs, platelets bind avidly to exposed subendothelium and to each other. The principal substrate for $\alpha_{IIB}\beta_3$ is fibrinogen, but the expressed complex can also bind vitronectin and von Willebrand factor. In terms of overall involvement in platelet physiology and pathology, $\alpha_{IIB}\beta_3$ appears to be the most important integrin receptor, while glycoprotein Ib–IX has that distinction amongst the nonintegrins. At high shear rates GPIb–IX is the primary receptor involved in platelet adherence to subendothelium, while at low shear rates $\alpha_{IIB}\beta_3$ serves that function. Thus, there is a physiologic basis for the redundancy of receptors and receptor complexes.

Receptor mobility: The morphology of platelet receptor interactions with surfaces has been difficult to visualize in the electron microscope because they occur at a molecular level beyond the resolution of available instruments. However, the use of immunofluorescent microscopy and immunocytochemical methods combined with electron microscopy have largely circumvented this problem. Fluorescence microscopy revealed that platelets in suspension exposed first to monoclonal antibodies against GPIIb–IIIa $\alpha_{IIB}\beta_3$ and then fluorescein tagged antiimmunoglobulin revealed a sequence of clustering, patching, capping and endocytosis similar to events observed on lymphocytes exposed to immune reactions[40–42]. Studies with fluorescein conjugated concanavalin A revealed a similar sequence of patching/capping on platelets.

Polley et al.[43] were probably the first to demonstrate movement of receptors on platelets at the ultrastructural level. Immunocytochemical methods combined with a membrane fragmentation procedure were used to demonstrate that thrombin treatment caused clustering or patching of GPIIb–IIIa receptors on platelet surfaces. Eisenberg

Cambridge Books Online © Cambridge University Press, 2009

and colleagues[44] used a replica technique to demonstrate that ADP induced ligand binding and fibrinogen receptor clustering on platelet surface membranes. Santoso and associates[45] demonstrated receptor patching on platelets exposed to monoclonal antibodies or incubated with concanavalin A at the ultrastructural level, confirming the observations made by immunofluorescence. Loftus and Albrecht[46] and, subsequently, Albrecht et al.[47–49] introduced the use of fibrinogen-coated, colloidal gold particles to study GPIIb–IIIa receptors on surface-activated platelets. Their findings suggested that the shape change and spreading caused by surface activation stimulated redistribution of GPIIb–IIIa receptors into caps on spread platelets.

Investigations by Hourdille et al.[50] have shown that thrombin causes spontaneous clearance of GPIb–IX from the platelet surface to channels of the open canalicular system. More recently, she and her colleagues[51] demonstrated that von Willebrand factor bound to glycoprotein Ib–IX is cleared from the platelet surface following activation by thrombin. The dynamic redistribution of major platelet surface receptors following surface activation and spreading has been confirmed and extended by Kieffer et al[52]. Thus, evidence coming from many investigations indicates that major integrin and nonintegrin receptors on platelets are mobile and undergo relocation, clustering, patching, capping and clearance following exposure to potent stimuli in suspension or after contact with foreign surfaces.

Work in our laboratory has confirmed many of the findings reported by others and added additional information to the knowledge of mobile receptors on platelet surfaces[53]. Initial experiments were carried out on platelets allowed to spread on carbon-stabilized, formvar-coated grids for 20 minutes and then exposed to fibrinogen-coated gold particles (Fgn/Au) for 5 minutes at 37 °C. Most cells retaining a discoid configuration failed to bind Fgn/Au, though some did (Fig. 3.16). The morphological difference between discoid cells expressing GPIIb–IIIa and those not binding the electron dense probe was not apparent. Early dendritic forms were covered by particles of Fgn/Au (Fig. 3.17). The distribution appeared random and discrete, though a suggestion of clustering may have been due to the convoluted organization of the platelet surface discussed above. On late dendritic cells manifesting early signs of pseudopod and cytoplasmic spreading, Fgn/Au particles moved away from the edges to the midlines of surface extensions and from them and the cell body toward the central region (Fig. 3.18).

The tendency of the Fgn/Au bound to $\alpha_{IIB}\beta_3$ receptors to move away from peripheral margins toward cell centres

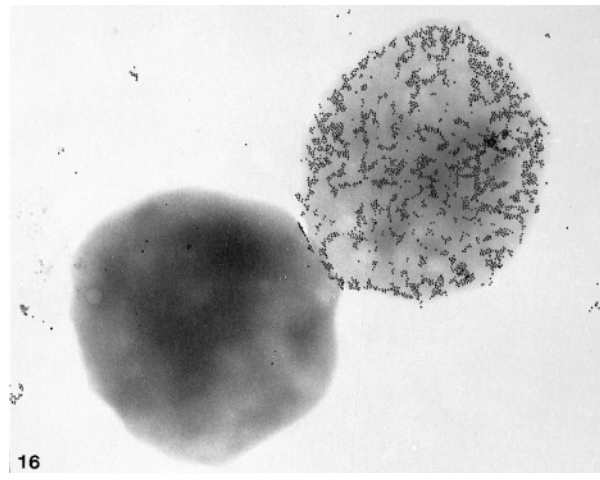


Fig. 3.16. Platelets incubated with 10^{-6} M cytochalasin E in suspension before contact with a formvar grid for 20 min and subsequent exposure to fibrinogen-coated gold (Fgn/Au) particles for 5′. Both cells have retained their discoid shape. However, only one platelet has expressed glycoprotein IIb–IIIa receptors and bound a significant number of Fgn/Au particles. Only a few receptors ligand complexes are apparent on the other discoid platelet (Mag ×16 000).

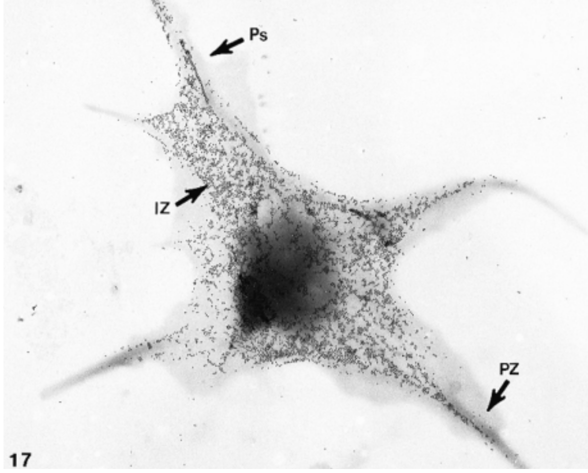


Fig. 3.17. Early dendritic platelet exposed to fibrinogen-gold (Fgn/Au) for 5 min after initial contact with a formvar-coated, carbon-stabilized grid for 20 min before fixation in glutaraldehyde. Fgn/Au spherules extend out onto pseudopods (Ps) and coat the intermediate zone (IZ) and central zone (CZ). Areas of the cell margin on pseudopods and along the peripheral zone (PZ) appear virtually free of gold particles (Mag ×12 000).

Cambridge Books Online © Cambridge University Press, 2009

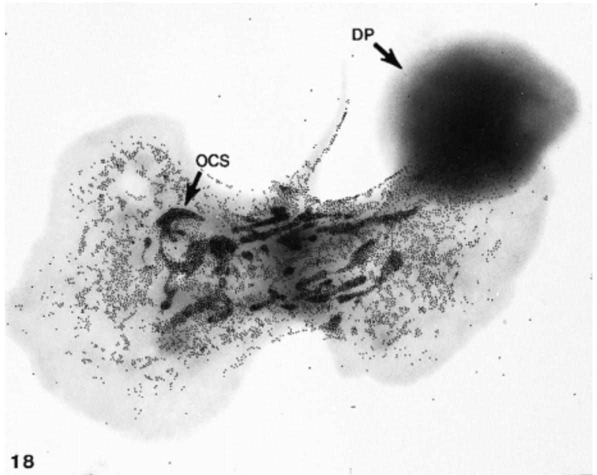


Fig. 3.18. Late dendritic platelet. Cell is from a sample prepared in the same manner as the cell in Fig. 3.17. Fgn/Au particles bound to GPIIb–IIIa receptors have moved from the peripheral border toward the platelet centre and into channels of the open canalicular system (OCS). A discoid, resting platelet has not expressed GPIIb–IIIa receptors and, as a result, has not bound Fgn/Au particles (Mag ×16 000).

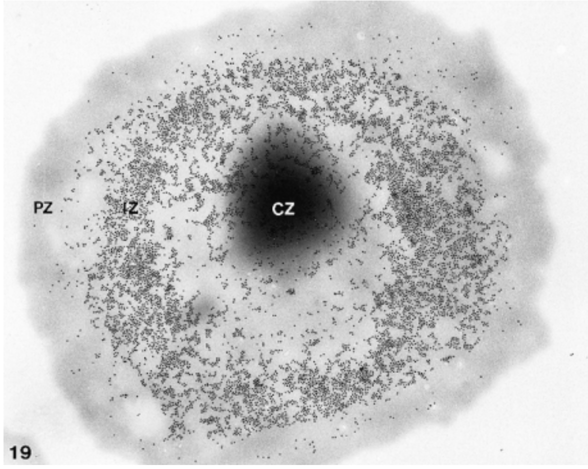


Fig. 3.19. Fully spread platelet exposed to Fgn/Au for 5 min. Electron-dense probes bound to GPIIb–IIIa receptors have moved from the peripheral zone (PZ) to form a concentrated ring in the immediate zone (IZ) around the central zone (CZ). The central area is nearly as devoid of receptor–ligand complexes as the peripheral zone (Mag ×18 000).

was more prominent on early spread and fully spread platelets[54]. Fgn/Au receptor complexes became concentrated in a ring located in the intermediate zone (Fig. 3.19). The central area of spread platelets was nearly as devoid of Fgn/Au probes for $\alpha_{IIB}\beta_3$ as the peripheral zone. Exposure

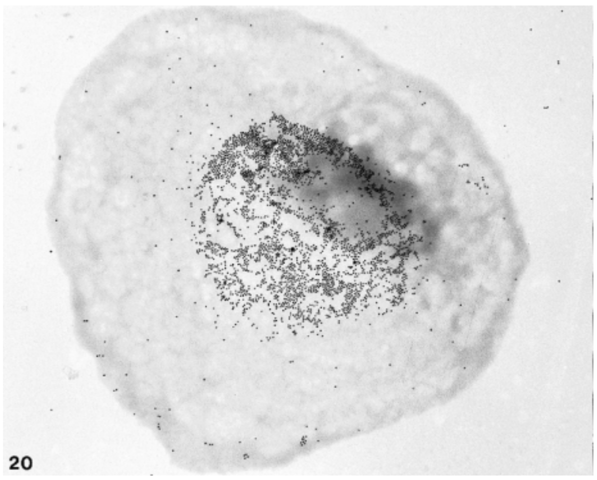


Fig. 3.20. Capping. Fully spread platelet exposed to Fgn/Au for 5 min, rinsed and then rested on a drop of Hank's balanced salt solution for 15 min at 37 °C. Fgn/Au bound to GPIIb–IIIa receptors have moved into a concentrated mass or cap on the central zone. The intermediate and peripheral zones are virtually devoid of Fgn/Au particles, suggesting that nearly all of the GPIIb–IIIa receptors have been translocated to the cell centre (Mag ×18 000).

of spread platelets to Fgn/Au for longer intervals of 10 to 30 minutes did not result in concentration of receptor–ligand complexes in the central zone. However, simply rinsing excess gold from the grid with Hank's balanced salt solution (HBSS) and allowing the grid to rest on a drop of HBSS for 10 minutes at 37 °C caused further central movement of Fgn/Au bound to $\alpha_{IIB}\beta_3$, resulting in cap formation[55] (Fig. 3.20). Precisely why this manoeuvre causes capping to occur when longer incubation fails remains obscure. It is possible that the Fgn/Au suspension inhibits further spreading of platelets during the 5-minute incubation, and that washing and resting on HBSS removes the inhibitory influence.

The appearance of the platelet with a central cap of Fgn/Au–$\alpha_{IIB}\beta_3$ complexes is striking. Intermediate and peripheral zones of the cells are virtually devoid of Fgn/Au particles, suggesting that nearly all of the $\alpha_{IIB}\beta_3$ receptors have been cleared to the cell centres. However, addition of another drop of Fgn/Au after capped cells are fixed results in edge to edge coverage of the peripheral and intermediate zones[56]. If capped cells are exposed to small latex spherules without prior fixation, these particles bind to the bare areas and move into a ring around the central cap of Fgn/Au–$\alpha_{IIB}\beta_3$ complexes, leaving the peripheral margin bare (Fig. 3.21). Rinsing the capped platelets ringed with latex particles, and subsequent exposure to a drop of Fgn/Au results in binding of the electron-dense probe for

Cambridge Books Online © Cambridge University Press, 2009

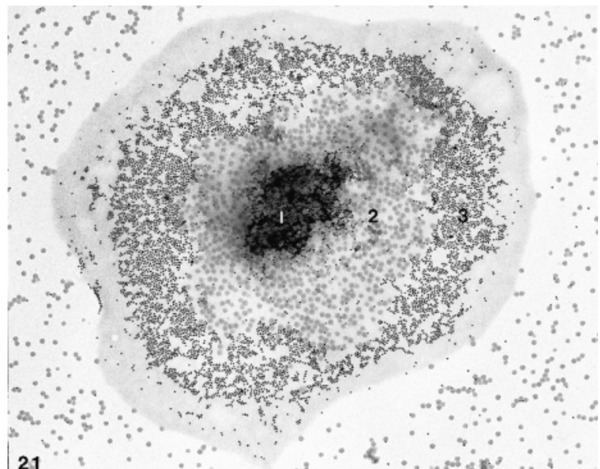


Fig. 3.21. Spread platelet exposed to Fgn/Au, rinsed and rested on HBSS, and then exposed in the same manner to small latex spherules, washed and arrested on HBSS and exposed again to Fgn/Au. The first wave of Fgn/Au–GPIIb–IIIa complexes are concentrated in a central cap (1). Latex spherules have concentrated into a ring around the cap (2) and the second wave of Fgn/Au has moved centrally into a ring around the latex (3). The peripheral margin is virtually clear of receptor–ligand complexes (Mag $\times$18 000).

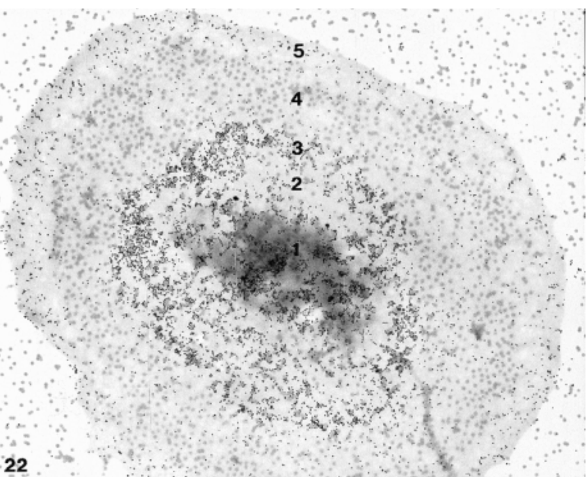


Fig. 3.22. The spread platelet in this example was exposed first to Fgn/Au (1) then to latex (2), then to Fgn/Au (3) followed by another incubation with latex (4), washed and fixed in 0.1% glutaraldehyde. Subsequently, the platelet was incubated in Fgn/Au (5) and examined in the electron microscope. The cap of Fgn/Au–GPIIb–IIIa complexes is surrounded by two rings of latex interspersed with two rings of Fgn/Au. Formation of the central cap of receptor–ligand complexes has not exhausted the supply of GPIIb–IIIa elsewhere on the exposed cell surface (Mag $\times$ 10 000).

$\alpha_{IIB}\beta_3$ to the barren area. The receptor–ligand complexes undergo central movement into a ring around the latex spherules, again leaving a bare peripheral margin. Repetition of the sequence resulted in capped cells with two rings of latex separating two of Fgn/Au–$\alpha_{IIB}\beta_3$ complexes (Fig. 3.22). Capping does not exhaust the supply of $\alpha_{IIB}\beta_3$ receptors on spread platelets. Yet, the origin of the $\alpha_{IIB}\beta_3$ receptors binding the second and third waves of Fgn/Au remains unclear. Evidence that they are being recycled from the central cap has not been convincing.

The major destination of Fgn/Au–$\alpha_{IIB}\beta_3$ complexes on spread platelets is not limited to the intermediate or central zones. About 5–10% of the spread platelets contain channels of the surface connected OCS filled with Fgn/Au bound to $\alpha_{IIB}\beta_3$ (Figs. 3.18, 3.23). Experiments to determine why more spread platelets did not contain OCS channels filled with Fgn/Au receptor complexes provided two answers, both of which appear to be correct[57]. First, most of the OCS channels in fully spread platelets had already been evaginated out onto the surface. Only a very large cell appeared less capable of completely evaginating the OCS, and was, therefore, much more likely to display Fgn/Au filled OCS channels. The second reason was that the spreading process produced sufficient tension, both horizontally and vertically, to seal off the residual OCS channels. Treatment of fully spread platelets after exposure to

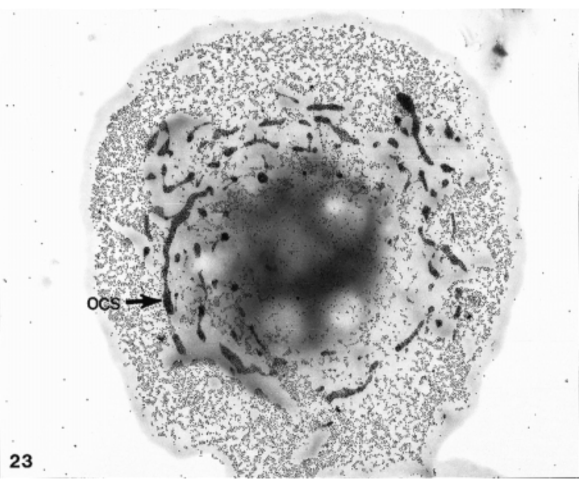


Fig. 3.23. Spread platelet exposed to Fgn/Au probes for 5 min. Receptor–ligand complexes have moved from the periphery toward the cell centre and into channels of the OCS (↑). The central area is more electron-opaque because it is the residual body. Few Fgn/Au markers for GPIIb–IIIa are present centrally (Mag $\times$15 000).

Cambridge Books Online © Cambridge University Press, 2009

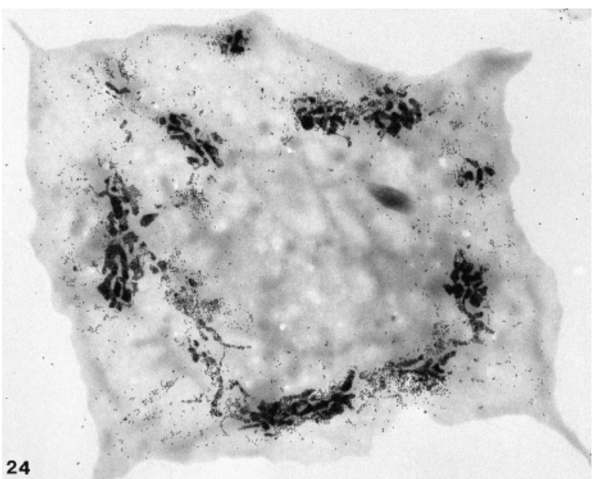


Fig. 3.24. Spread platelet exposed to Fgn/Au for 5 min and then to 10^{-4} M cytochalasin B. The agent has caused breakdown of new actin filaments near the cell periphery. Central movement has been reversed and Fgn/Au-GPIIb–IIIa complexes have moved back toward the periphery. Relaxation of the cell periphery by cytochalasin E has exposed residual short channels of OCS. The receptor–ligand complexes are deposited in the short OCS channels (Mag ×11 000).

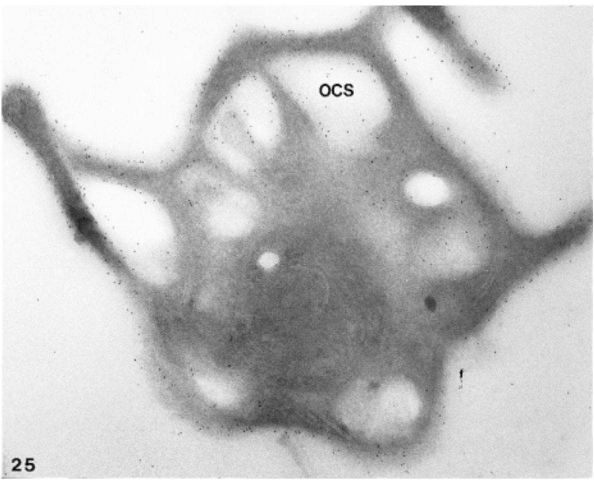


Fig. 3.25. Immunolocalization of GPIb in a frozen thin section obtained from platelets exposed to 1 U/ml of thrombin for 10 minutes in suspension. Section was incubated with a polyclonal antibody against glycocalicin followed by protein A-gold (5 nm). The cell has become irregular after exposure to thrombin and channels of the OCS (↑) are dilated. Exposed surface membrane and membrane lining channels of the OCS are evenly labeled with gold particles indicating sites of GPIb (Mag ×54 000).

Fgn/Au first with cytochalasin B resulted in softening of the peripheral margins due to new actin filament disassembly and appearance of Fgn/Au–receptor complexes in short segments of OCS near the cell margin (Fig. 3.24). Uptake of Fgn/Au bound to $\alpha_{IIB}\beta_3$ into OCS channels was always greater in early dendritic platelets than in spread cells, just as it is in thrombin-treated platelets in suspension. Thus, the OCS appears to be the principal destination for receptor–ligand complexes on surface-activated platelets, just as it is on suspension-activated cells[58,59].

Other studies have examined the distribution of GIb–IX and $\alpha_{IIB}\beta_3$ receptors on suspension and surface-activated platelets before and after exposure to 1 U/ml of thrombin for 10 minutes[60]. Frozen thin sections prepared from fixed suspension-activated platelets or grids containing fixed surface-activated platelets were stained with specific antibodies to GPIb–IX (anti-glycocalicin) and $\alpha_{IIB}\beta_3$ (AP_2 or 7E3), and incubated with the corresponding gold-coupled secondary antibody. GPIb–IX and $\alpha_{IIB}\beta_3$ were present in the open canalicular system of resting and, more prominently, in dilated OCS channels of cells activated by thrombin in suspension (Figs. 3.25, 3.26). On surface-activated platelets more intense labelling for GPIb–IX was observed along pseudopods of dendritic cells while $\alpha_{IIB}\beta_3$ receptors were slightly increased over the peripheral zone (Figs. 3.27, 3.28). The observations support previous investigations

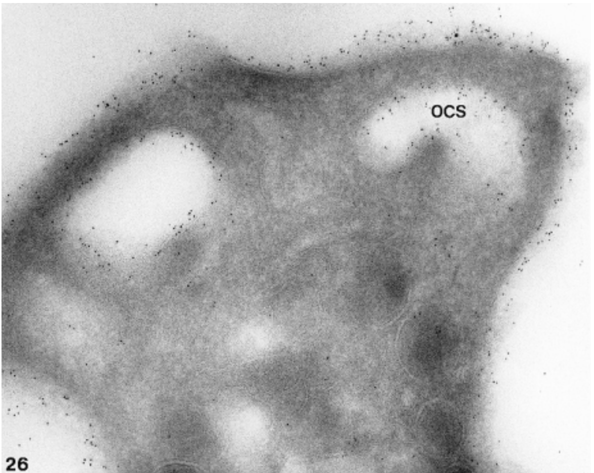


Fig. 3.26. Immunolocalization of GPIIb/IIIa in another frozen thin section from the sample shown in the previous illustration. In this section, a monoclonal antibody against GPIIb/IIIa (AP-2) was used followed by a goat antimouse IgG coupled to gold (5 nm). Gold particles indicating sites of GPIIb/IIIa are evenly distributed on the external membrane and membranes lining channels of the OCS (Mag ×54 000).

Cambridge Books Online © Cambridge University Press, 2009

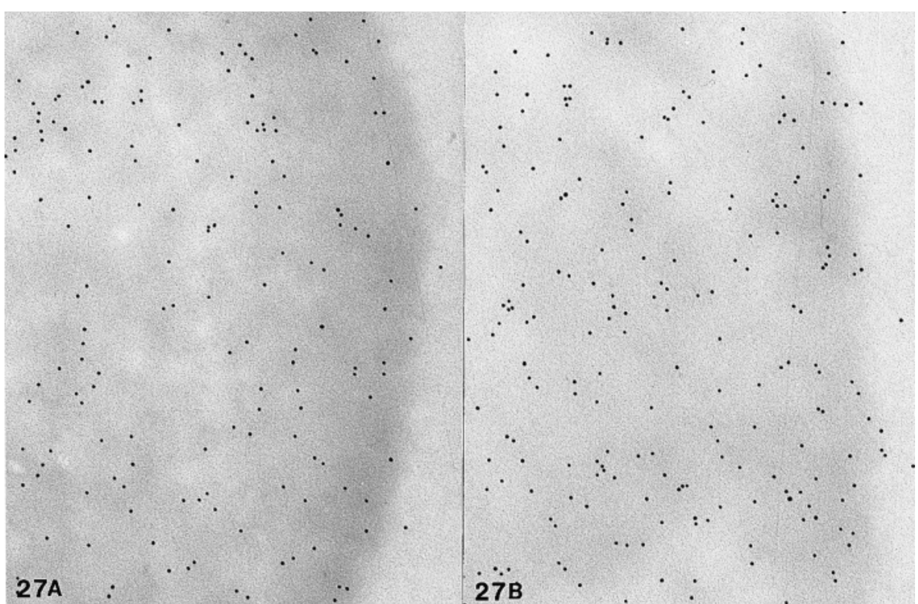


Fig. 3.27(*a*) and (*b*). Immunolocalization of GPIb on dorsal surface of fully spread platelets. The cells were allowed to interact with formvar coated grids for 20 minutes and for an additional 10 minutes to (*a*) buffer or (*b*) to thrombin, 1 U/ml. GPIb receptors are evenly dispersed over platelet surfaces from edge to edge before (*a*) and after (*b*) exposure to thrombin (Mag ×50 000).

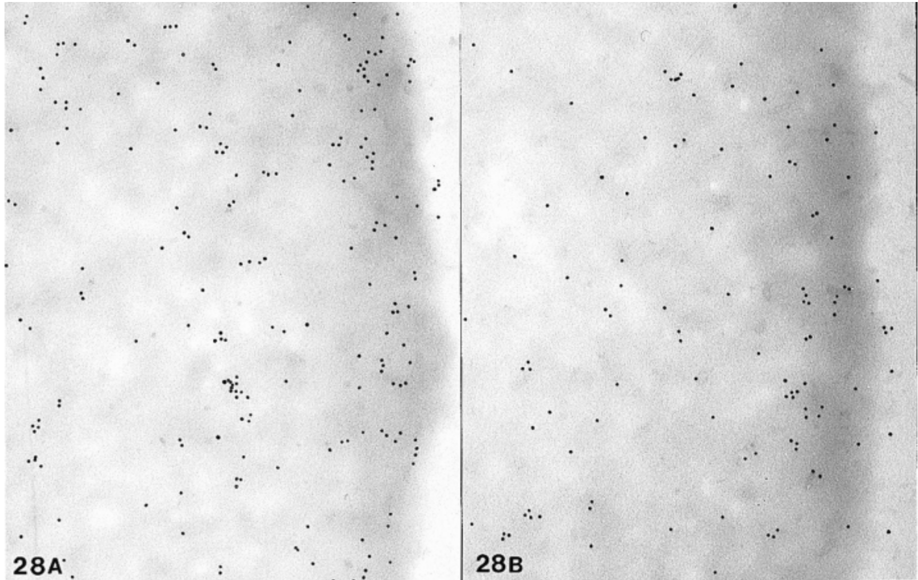


Fig. 3.28(*a*) and (*b*). Immunolocalization of GPIIb/IIIa on fully spread platelets (20 min) allowed to interact with (*a*) buffer or (*b*) thrombin, 1 U/ml, for an additional 10 min. The distribution of gold particles indicating sites of GPIIb/IIIa are essentially the same before and after exposure of spread platelets to thrombin (Mag ×50 000).

Cambridge Books Online © Cambridge University Press, 2009

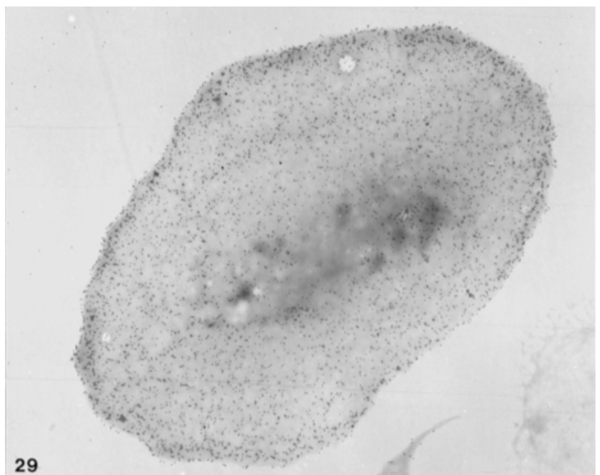


Fig. 3.29. Surface-activated, fully spread platelet fixed in 0.01% glutaraldehyde before exposure to Fgn/Au. Particles of Fgn/Au are evenly dispersed over the entire platelet from edge to edge. Thus, fibrinogen receptors marked by Fgn/Au do not spontaneously reorganize during the shape change stimulated by surface activation (Mag ×12 000).

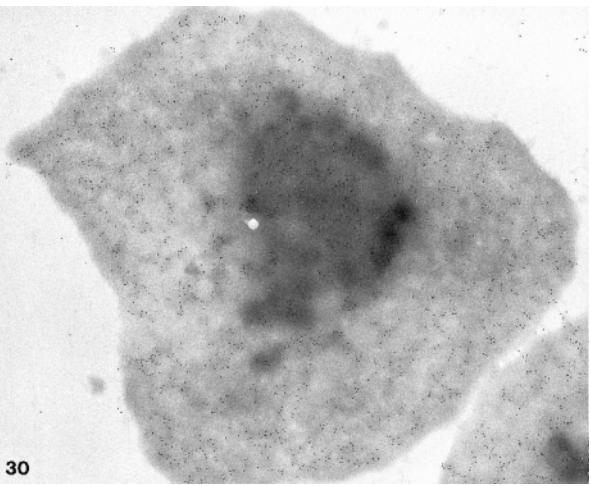


Fig. 3.30. Platelet from sample of washed cells exposed to 0–5 U/ml of thrombin in suspension for 10 min and spread on formvar grid for 20 min before fixation. The fixed cells were incubated with ristocetin-activated human plasma and then stained by anti-vWF and PAG_{10}. Multimers of vWF cover the cell surface from edge to edge (Mag ×16 500).

demonstrating that shape change and spreading do not cause spontaneous clearance of $\alpha_{IIB}\beta_3$ receptors, and also indicate that GPIb–IX receptors are resistant to spontaneous clearance from exposed membranes of surface- or suspension-activated platelets before or after exposure to thrombin.

The experiments described here, and in other work from our laboratory, strongly support the concept that major integrin and non-integrin receptors on platelet surfaces are mobile[61]. However, the receptors are not spontaneously cleared from the cell surface by exposure to thrombin in suspension, or treatment with this agent after the cells are already activated on surfaces[62,63] (Figs. 3.29, 3.30). $\alpha_{IIB}\beta_3$ receptors may be cleared to channels of the OCS from exposed membranes of surface- and suspension-activated platelets, but are by no means exhausted by the process. Rather, new populations of the fibrinogen receptors are ready to react, even though substantial numbers appear to have formed caps with the ligand or concentrated in channels of the OCS. GPIb–IX also resists spontaneous clearance on surface and suspension-activated platelets[64] (Figs. 3.31, 3.32). It remains to be determined whether clearance of GPIb–IX with von Willebrand factor to channels of the OCS following exposure of platelets to thrombin exhausts the supply of the receptor on exposed surfaces, or that they are replaced by new populations capable of interacting with vWF.

It should be appreciated, however, that nearly all the

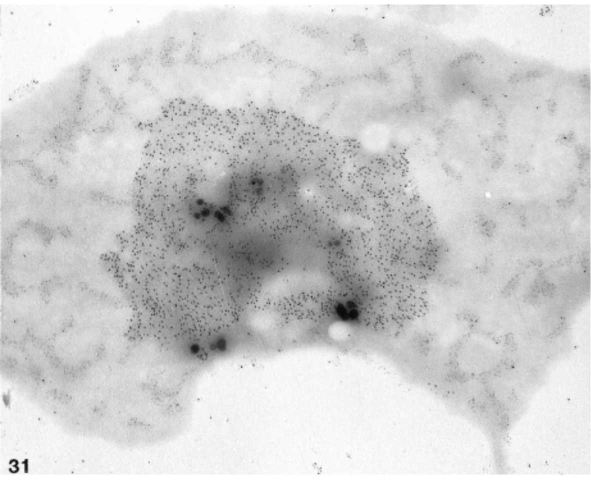


Fig. 3.31. Platelet from sample of washed cells treated with thrombin, 2 U/ml, in suspension for 10 min, spread on a grid for 20 min, incubated with bovine vWF for 15 min, followed by anti-vWF antibody for 20 min. The cells were fixed with 0.01% glutaraldehyde and stained with PAG_{10}. The grid was then washed, exposed again to bovine vWF, combined with anti-vWF antibody, and stained with PAG_5, before refixation. The cap of vWF multimers stained by PAG_{10} is concentrated in the cell centre. Multimers of vWF from the second exposure to bovine plasma are stained by PAG_5 and extend from the central cap to the cell margin (Mag ×23 000).

Cambridge Books Online © Cambridge University Press, 2009

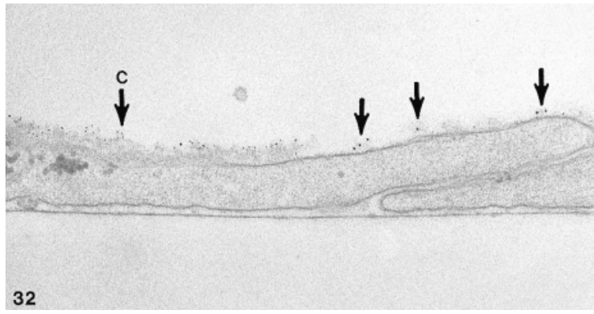


Fig. 3.32. Thin section of a platelet treated in the same manner as the cell in the previous illustration, except that it was spread on glass. PAG_5 was used for the first exposure to vWF, and PAG_{10} for the second. A cap (C) consisting of vWF, vWF antibody and PAG_5 covers the central area of the platelet surface. Multimers extending from the cap to the cell edge are stained by PAG_{10} (↓) (Mag ×40000).

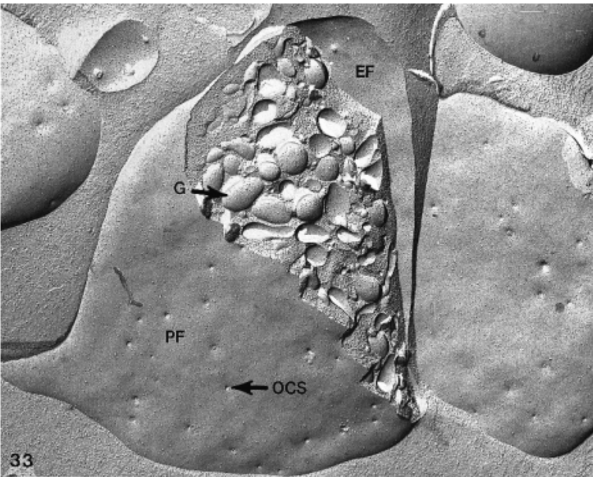


Fig. 3.33. Unit membrane. Platelets from a sample of citrated PRP fixed in glutaraldehyde, infiltrated with glycerol, frozen in liquid nitrogen, and fractured in a freeze–etch device. A carbon–platinum replica of freeze–fractured surface reveals many details of platelet unit membrane and membrane systems. Freeze–fracture splits the lipid bilayer exposing the outer surface of the inner leaflet (PF) or the inner side of the outer layer (EF). Each face is covered by randomly dispersed particles varying slightly in size. Communications between channels of OCS and cell surface appear as small indentations on PF and interiorly directed protuberances on EF. Granule (G) membranes in platelet cytoplasm are also coated with small particles (Mag ×26000).

experiments reported thus far have focused on the translocation of major non-integrin and integrin receptors bound to movable ligands across the cell surface to channels of the OCS. Yet, the ligands to which platelets adhere on damaged vascular surfaces are not mobile[61]. Therefore, mobile receptors must have some other function than to clear the surface of ligands, thereby eliminating their own availability for hemostasic reactions. Indeed there is. Experiments indicate that mobile receptors are critical to the process of platelet tethering and spreading on injured vessels, foreign surfaces, fibrin strands and each other. The mobile receptors, primarily GPIb–IX and $\alpha_{IIB}\beta_3$, bind to ligands exposed in the subendothelium and form stable complexes. The platelet cannot move these rigid receptor ligand complexes to channels of the open canalicular system. Instead, the platelet surface and membranes lining OCS channels move through the stable receptor–ligand complexes driven by assembling actin in order to achieve maximum spreading. While the major function of the receptors is to secure platelet adhesion, the reason why they are mobile is to facilitate extension of the surface membrane over as much surface area as possible. If the platelet had rigid, immobile receptors, adhesion of discs to a surface could not be followed by spreading.

Unit membrane

The platelet unit membrane provides a physico-chemical separation between intracellular and extracellular constituents and processes. Important components of the platelet unit membrane are the Na/K ATPase and the anion pump, which help maintain the appropriate transmembrane ionic gradients. Examination of trilaminar membranes of the peripheral zone in thin sections of platelets has failed to reveal any apparent differences from unit membranes of other blood cells. Replicas of freeze–fractured platelets, however, reveal significant variations in the number and distribution of intercalated particles[65] (Figs. 3.7, 3.33). The outside of the inner face (P face) and the interior of the outer lamellae (E face) of the split lipid bilayer of red cell membranes are coated with particles, whereas intercalated particles on similarly exposed surfaces of freeze-fractured platelets are few and randomly dispersed. Intramembranous particles are present in largest numbers on the P face of red cells, but are most numerous on the E face of platelet split lipid bilayers. It is likely that the intercalated particles represent portions of transmembrane proteins buried in lipid constituents.

Schick, Kurica, and Chacko[66] have used a nonpenetrating membrane probe, 2,4,5-trinitrobenzenesulfonate, in an attempt to localize phospholipid groups in the platelet surface membranes. Results of their study suggest that the bilayer is asymmetric with regard to its phospholipid composition. Phosphatidylserine does not appear to be part of the exposed surface, whereas a significant proportion of the phosphatidylethanolamine was labelled by the mem-

Cambridge Books Online © Cambridge University Press, 2009

brane probe in activated cells, particularly in thrombin-treated platelets. Thus, even though studies of chemical composition have not indicated striking differences in platelet lipids compared to other blood cells, the organization of specific molecules in the bilayer appears to impart unique properties to the surface.

Despite the absence of distinguishing physical characteristics, the platelet unit membrane is considered to be of critical importance in the physiology of hemostasis. During platelet aggregation and transformation, the cells provide an essential substance for acceleration of blood coagulation[67]. This substance, often referred to as platelet factor 3, is believed to reside in the lipoprotein-rich unit membrane. The mechanism by which the membrane phospholipid becomes exposed to serve as a catalytic surface for plasma proteins is not known with certainty.

The ability of the platelet unit membrane to maintain ionic gradients may rival its importance as a surface for accelerating coagulation. Simons and her colleagues have used fluorescent probes to follow changes in the pH gradient across platelet surface membranes[68]. Their studies suggest that neutralization of the transmembrane pH gradient is the earliest event to occur after exposure of platelets to an activating agent.

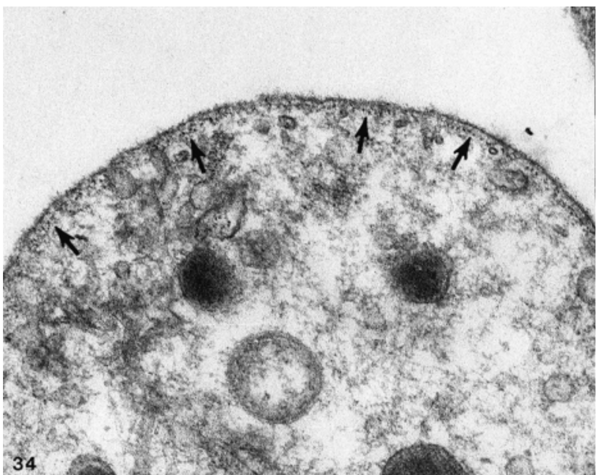


Fig. 3.34. Submembrane area. Filaments are the major constituents of this region but are difficult to resolve in well-fixed platelets because of the density of the cytoplasm (see Fig. 3.2). However, the submembrane filaments are revealed as a nearly continuous layer (*arrows*) just inside the surface following exposure of the cells to osmotic or chemical shock (Mag × 58 000).

Submembrane region

The area lying just under the unit membrane of the platelet surface has special characteristics that impel its inclusion with other structural components of the peripheral zone. It has been evident for some time that organelles inside the matrix of unaltered platelets never contact the cell wall. The nature of the special barrier under the cell surface has been unclear, but the submembrane area has been shown to contain a relatively regular system of filamentous elements[69] (Fig. 3.34). Submembrane filaments are obscured by the dense matrix of the sol–gel zone, but they can be seen peripheral to the circumferential band of microtubules in discoid cells. Treatment with chemical or osmotic shock renders them visible as a distinct system under the unit membrane. Submembrane filaments are physically similar to actin microfilaments[70] (Figs. 3.35, 3.36). However, it is possible that they are the physical equivalents of filamin, α-actinin, or some other anchoring protein for the platelet contractile system. The close association of submembrane filaments with the cell surface is the distinguishing feature of this system and the reason that it is considered part of the peripheral zone. Functionally, the submembrane filaments may cooperate with circumferential microtubules to maintain platelet discoid shape, contribute to the extrusion and stabilization of pseudopods, and interact with other elements of the

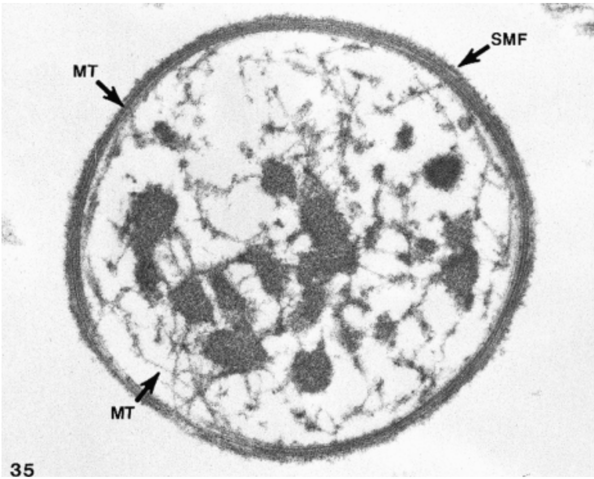


Fig. 3.35. Suspension cytoskeleton. Thin section of a platelet fixed after detergent extraction in the presence of lysine and phalloidin. The microtubule (MT) is well preserved. A rough amorphous coat is evident on its outer surface. Remnants of alpha granules are suspended in a matrix of actin microfilaments (MF) resistant to detergent extraction. Submembrane filaments (SMF) form a fine fuzz on the outer coil of the circumferential microtubule (Mag ×29 000).

Cambridge Books Online © Cambridge University Press, 2009

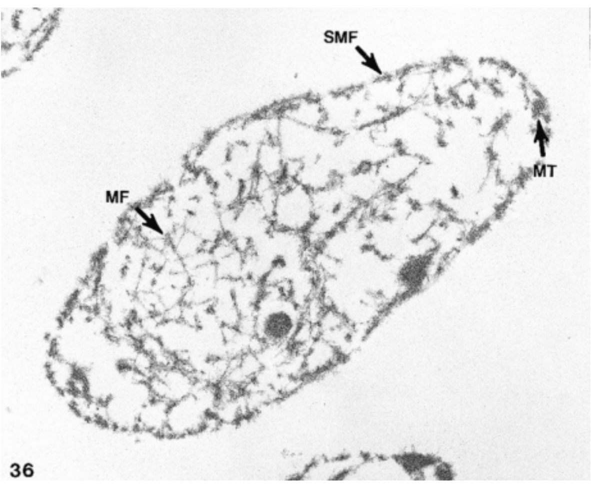


Fig. 3.36. Suspension cytoskeleton. Cross section of a discoid platelet cytoskeleton. The cell is from a sample of citrated-PRP treated with taxol (10^{-4} M) before extraction. Microtubule (MT) profiles are evident at the polar ends of the cell. A meshwork of the microfilaments (MF) replaces the cytoplasmic matrix. Although the surface membrane is gone, a fine amorphous layer of submembrane filaments (SMF) remains in its place (Mag × 29 000).

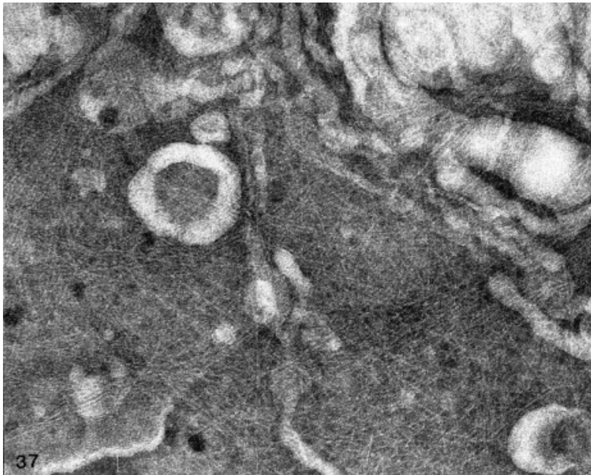


Fig. 3.37. Sol–gel zone. Cytoplasm of a negatively stained whole-mount of a spread platelet. The matrix of the cell is filled with an irregular meshwork of microfilaments. The 50 Å fibres are indistinguishable from the subfilaments of microtubules and submembrane filaments (Mag × 66 000).

platelet contractile mechanism to effectuate viscous metamorphosis and clot retraction[19,28,70].

Sol–gel zone

Early light and phase contrast microscopic studies revealed the presence of organelles inside blood platelets, but the transparency of the matrix prevented recognition of other structural components. The formed bodies were believed to be dispersed in a fluid suspension or salt solution, the clarity of which suggested the term hyaloplasm. Although it is now apparent that the internal environment of platelets is made up of many structural elements, the idea that these components are in solution or suspension remains prevalent. When thin sections of platelets were examined in the electron microscope the internal matrix appeared to consist of an irregular meshwork of fibrous material in which were embedded formed organelles. It was suggested that the matrix was an artefact created by precipitation of protein from solution during fixation. To some extent this concept was correct. However, the asymmetric shape of living platelets and separation of granules from one another visible in the light microscope should have suggested that the hyaloplasm must be extremely viscous. Since high internal viscosity results to a large extent from molecular interaction, it was predictable that protein polymers should be present in platelet hyaloplasm.

The introduction of glutaraldehyde fixation at 37 °C prior to treatment with osmic acid at low temperature revealed that the matrix of the platelet was a dense mat of fibrous elements[12,71] (Figs. 3.35–3.37). At first, these elements were thought to be artefacts of fixation, but studies by the negative stain whole-mount method revealed that fibers of various types were also present in unfixed platelets[4]. Subsequent investigations have shown that changes in the state of polymerization and movement of the fibrous components of the matrix are intimately related to support of platelet discoid shape and to internal contraction. Since it became clear that the matrix inside platelets resembles a gel, it seemed appropriate to focus on this characteristic by renaming the hyaloplasm and calling it the sol–gel zone[13].

Microtubules

At least three systems of fibres are present in the matrix of the platelet, including submembrane filaments, microtubules, and microfilaments. The most prominent of the three systems is the circumferential band of microtubules[72–74] (Figs. 3.1–3.4). Cross-sections of fixed platelets reveal microtubules as a group of 8–24 circular profiles, each approximately 25 nm in diameter, at the polar ends of the lentiform cell[29]. When platelets are sectioned in the equatorial plane, the bundle of tubules is apparent just under the cell wall along its greatest circumference. Circumferential tubules are always slightly separated from

Cambridge Books Online © Cambridge University Press, 2009

each other, though small bridges between tubules can occasionally be identified[17]. Although the bundle lies close to the cell wall it never appears to contact it. The space between the tubules and the surface is often occupied by submembrane filaments cut in cross-section[69]. In the unaltered circulating platelet evidence suggests that tubulin, the protein constituent of microtubules, exists almost exclusively in the polymerized (microtubule) form[75].

Study of circumferential band microtubules by the negative stain whole-mount method revealed their substructural characteristics[76]. Each tubule is in itself a fibrous system. When fully spread, the tubules consist of 12–15 subfilaments. The subfilaments lie in parallel association with a centre-to-centre spacing of approximately 70 Å. Each individual filament appears to consist of globular subunits stacked in an offset manner to yield a helical twist. At points where spread tubules are broken, individual subfilaments can be seen spreading out from the fracture. This finding suggests that subfilaments are more stable than the microtubule, and polymerization of subfilaments may precede their parallel association into the tubule form.

The location of the circumferential band of microtubules in the equatorial plane just under the cell wall in discoid platelets suggested its participation in cytoskeletal support (Figs. 3.2, 3.4). Further proof for this role came from several experimental approaches. Platelets were long known to lose their discoid form and become irregular and relatively spherical when exposed to low temperature for brief periods[77]. Discoid shape was restored if the chilled cells were warmed to 37 °C. The loss of discoid form was associated with disappearance of the circumferential band of microtubules, and recovery with reformation of the bundle in its usual position under the cell surface[78]. Colchicine, vincristine, and vinblastine, agents that inhibit mitosis by disassembling or preventing formation of microtubules, were also found to dissolve platelet microtubules[79]. The disappearance of the circumferential band of microtubules in platelets treated with these agents resulted in loss of platelet discoid shape. These studies strongly supported the concept that circumferential microtubules were involved in maintaining platelet lentiform appearance.

Other experiments, however, were not as easily explained. For example, when platelets were exposed to ethylenediamine tetracetic acid (EDTA) they lost their discoid shape and became 'spiny spheres'[77]. Yet, the band of microtubules remained intact and organelles were randomly dispersed within it[80]. Thus, shape change could take place in platelets despite persistence of the peripherally located circumferential coil of microtubules. However, if the blood was collected in citrate anticoagulant first, then the platelets washed and resuspended in buffer containing EDTA, cell discoid shape and circumferential microtubule coils were retained, though the effects of EDTA on membranes were the same as in platelets anticoagulated with EDTA alone. Therefore, the effect of EDTA on platelet shape change was due to interaction with exposed membranes of the surface and OCS, and not on the microtubule coils[81]. The mechanism by which EDTA produces this effect on platelet shape remains unknown.

The bovine platelet also raises a question regarding the central role of circumferential MT in cytoskeletal support of discoid shape[82]. Bovine platelets must be exposed to colchicine or vinca alkaloids for at least 1 hour longer than human cells to disassemble the microtubule rings. Chilling requires up to 2 hours to dissolve bovine platelet microtubules, and their removal does not cause loss of discoid shape[83]. Even after disappearance of the rings in bovine platelets the cells remain discoid and just as resistant to aspiration into a micropipette as untreated, control platelets. The basis for these differences in human and bovine platelets appears due to variations in their intrinsic anatomy and functional expression[84]. A peripheral arrangement of assembled actin filaments plays a role in supporting the discoid shape of bovine platelets. As a result, both disassembly of microtubules by colchicine and prevention or reversal of actin filament assembly by cytochalasin B were required to cause bovine platelets to lose their discoid form[82].

Perhaps the strongest arguments supporting a major role for actin filaments, rather than microtubules, supporting the discoid form of human platelets were raised by the work of Hartwig and his colleagues[85,86]. Their work indicated that the spectrin-reinforced surface membrane cytoskeleton and the actin-rich cytoplasmic cytoskeleton are responsible for maintaining discoid form, for shape change caused by surface or suspension activation, and for recovery when stimulation is incomplete. This concept has led to a new proposal to explain the mechanism involved in the shape changes induced in platelets by chilling and rewarming[87]. Exposure to low temperature caused severing of actin filaments associated with the surface membrane cytoskeleton. Cold also caused an elevation of cytoplasmic calcium, activation of gelsolin, uncapping and severing of established cytoplasmic actin filaments, formation of multiple nucleation sites, and rapid assembly of new actin filaments. The cooled platelets lost their discoid form, became swollen in appearance, and extended filopodia and lamellipodia. Rewarming to 24 to 37 °C did not restore the irregular, cold platelets to their resting, lens-like

Cambridge Books Online © Cambridge University Press, 2009

form. Rather, the new actin filament bundles formed in the cold underwent reorganization resulting in elimination of pseudopodia and formation of irreversible spheres.

This challenge to the concept placing circumferential microtubules as the major cytoskeletal system for maintaining platelet discoid shape was retested in our laboratory using the chilling-rewarming model together with microtubule stabilizing (Taxol) and destabilizing agents[88]. Washed platelet samples were rested at 37 °C and chilled to 4 °C; chilled and rewarmed to 37 °C for 60 minutes; or chilled, rewarmed, and exposed to the same cycle in the presence or absence of vincristine or Taxol and fixed for study by disseminated interference phase contrast microscopy and electron microscopy. Rhodamine phalloidin and flow cytometry were used to measure changes in actin filament assembly. Chilling caused loss of disc shape, pseudopod extension, disassembly of microtubule coils, and assembly of new actin filaments. Rewarming resulted in restoration of disc shape, pseudopod retraction, disassembly of new actin filaments, and reassembly of circumferential microtubule coils. Vincristine converted discoid platelets to rounded cells that extended pseudopods when chilled and retracted them when rewarmed, leaving spheres that could undergo the same sequence of changes when chilled and rewarmed again. Taxol prevented cold-induced disassembly of microtubules and limited pseudopod formation. Rewarming caused retraction of pseudopods on Taxol-treated, discoid cells. Cytochalasin B, an agent that blocks new actin filament assembly, alone or in combination with Taxol, inhibited the cold-induced shape change but not dilation of the open canalicular system. Rewarming eliminated open canalicular system dilation and restored lentiform appearance. The results indicate that microtubule coils are the major structural elements responsible for disc shape and its restoration after submaximal stimulation or rewarming of chilled platelets.

Questions may remain after these investigations, but they have been largely resolved by study of a child with a new bleeding syndrome[89]. The patient has a prolonged bleeding time, a history of easy bruising since infancy and abnormal platelet aggregation despite normal levels of GPIb–IX and GPIIb–IIIa receptors. Dense body frequency in his platelets was normal, indicating absence of platelet storage pool deficiency. Light and electron microscopy revealed that his platelets were spherical in form. Thin sections of his spherocytes revealed an absence of MT and MT coils in 98% of the cells. The 2% that contained microtubule coils were discoid. Immunofluorescence microscopy with an antibody against tubulin, the protein precursor of microtubules, revealed amounts similar to normal platelets, but none was assembled into tubules or coils. Addition

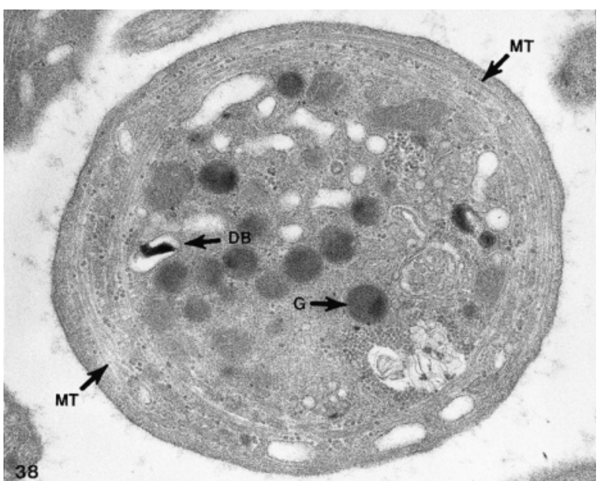


Fig. 3.38. Sol–gel zone. The platelet in this example has been sectioned in the equatorial plane and contains normal organelles including dense bodies (DB) and granules (G). A circumferential bundle of microtubules (MT) is apparent just under the cell surface. The bundle probably consists of a single tubule wrapped many times on itself like coils of a lariat. In this cell it forms several complete circles (Mag ×28 000).

of Taxol, an agent that stabilizes microtubules or stimulates their assembly, caused formation of microtubules and MT coils in his platelets and their conversion to discs. Results of the studies in this patient demonstrate that the circumferential coil of microtubules is the major cytoskeletal support system responsible for maintaining the discoid shape of human platelets (Fig. 3.38).

Microfilaments

Microfilaments constitute the second system of fibres in the platelet sol-gel zone. They appear more numerous than other polymers and are so concentrated in the matrix of unaltered platelets that they cannot be resolved in thin sections[12,90] (Figs. 3.35–3.37). However, they can be identified readily in whole mounts of spread platelets and in thin sections of platelet pseudopods. The state of microfilaments in unaltered platelets is not yet clear[28,75]. Subunit proteins of microfilaments exist primarily in a pregel, resembling the viscous state of hemoglobin molecules in intact erythrocytes. Polymerization into filaments may be a constant or alternating process, or may occur only as a result of stimulation during the hemostatic reaction of platelets. Whichever the case may be, it is apparent that the organization of the platelet internal matrix is governed by the balance of sol–gel transformation. Although many other components may be present in the sol–gel zone, microfilaments are the primary structural constituents involved in

Cambridge Books Online © Cambridge University Press, 2009

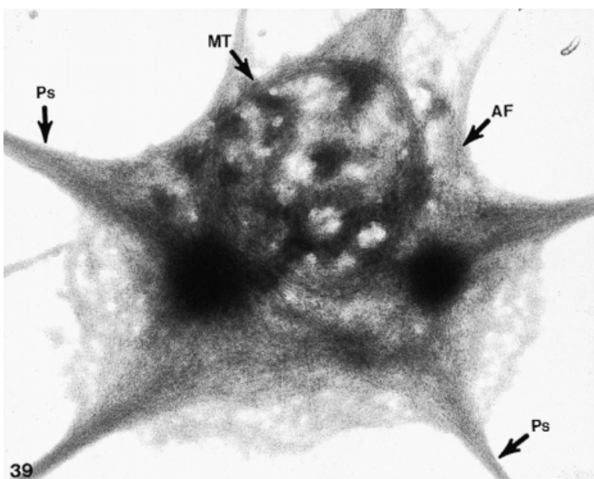


Fig. 3.39. Cytoskeleton of spread dendritic platelet. Surface stimulation has caused this cell to extend long, relatively rigid pseudopods (Ps). The spike-like extensions are filled with parallel bundles of actin microfilaments (AF). A loosely interconnected network of randomly arranged actin filaments fills spaces between the spike-like pseudopods. The circumferential microtubule (MT) is constricted and some of its free ends radiate into the cytoplasm and pseudopods (Mag ×23 000).

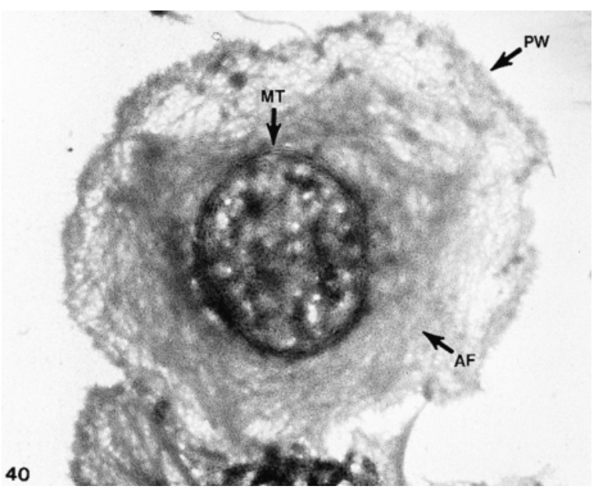


Fig. 3.40. Spread platelet cytoskeleton prepared from citrated-PRP. Coils of the circumferential microtubule (MT) are constricted in the cell centre. Actin microfilaments form a peripheral weave at the cell border and are organized in concentric layers around the MT coil (AF) (Mag ×23 000).

activation induced shape change and pseudopod formation[28,29,70,85,86] (Figs. 3.39, 3.40).

Organelle zone

The formed elements inside platelets have been of interest to investigators for many years. It was impossible to determine whether the cells contained one or several types of organelles in the light microscope, but early workers observed the disappearance of granules during hemostatic reaction and suggested that they contained secretory products important in platelet hemostatic function[5,91,92]. Electron microscopy revealed several types of organelles in the cytoplasm of platelets (Figs. 3.1–3.4). Mitochondria were easily differentiated from other structures by the plication of their internal membranes into cristae. Electron-dense bodies were distinctive because of their intensely opaque internal content, which was often separated from the enclosing membrane by a clear space[93]. Granules were the most numerous of the organelles in platelet cytoplasm and clearly different from mitochondria and electron-dense bodies.

Supporting the possibility that these organelles might be heterogenous were differences in the size of platelet granules; the electron opacity of their internal content; the presence of structures similar to microtubules in some, but not in others; and a zone of increased density resembling a nucleoid in many, but not in all. Investigations in several laboratories have strengthened this concept. Breton-Gorius and Guichard[94], employing a cytochemical technique that detects the peroxidatic activity of catalase, have shown that some of the small granules in platelets stain for this enzyme and resemble peroxisomes found in many other tissues. Bentfield and Bainton[95] have demonstrated that some vesicular elements contain acid phosphatase and aryl sulfatase, whereas granules do not. Da Prada et al.[96] have used biochemical and ultrastructural techniques to distinguish two populations of granules that can be separated by ultracentrifugation in urografin gradients. One population contains only platelet factor 4 and fibrinogen, whereas the other possesses only acid hydrolases. Thus, the organelle zone of normal platelets is more complex than was thought in early years. In addition to mitochondria, electron-dense bodies, and glycogen, it contains granules, vesicles, peroxisomes, lysosomes, and glycosomes.

Investigations on two patients with the grey platelet syndrome (GPS) have shed additional light on the chemical constituents localized within the several different types of granules of the organelle zone[97,98]. The two patients with the disorder have giant platelets. Thin sections reveal normal numbers of mitochondria and dense bodies but a marked reduction in granules. Levels of serotonin and adenine nucleotides were found to be within normal limits, as would be expected since the dense body content

Cambridge Books Online © Cambridge University Press, 2009

is normal. The catalase activity is normal and some of the granules stain for this enzyme, suggesting that peroxisomes are not abnormal in the GPS. Measurements of acid phosphatase, *N*-acetyl glucosaminidase and α-glucuronidase are normal, indicating that the majority, if not all of the few granules present are lysosomes. Thus, our studies suggested that GPS platelets are virtually devoid of α-granules and should be deficient in products confined to these organelles.

It seemed reasonable, therefore, to use GPS platelets to search for products normally present in α-granules. These studies have shown that GPS platelets are markedly deficient in thrombin-sensitive protein, α-thromboglobulin, fibrinogen, platelet factor 4, and the mitogenic factor. Thus, the use of the abnormal platelets has helped to confirm the existence of separate granule populations in platelets and permitted characterization of the specific chemical substances associated with α-granules[98].

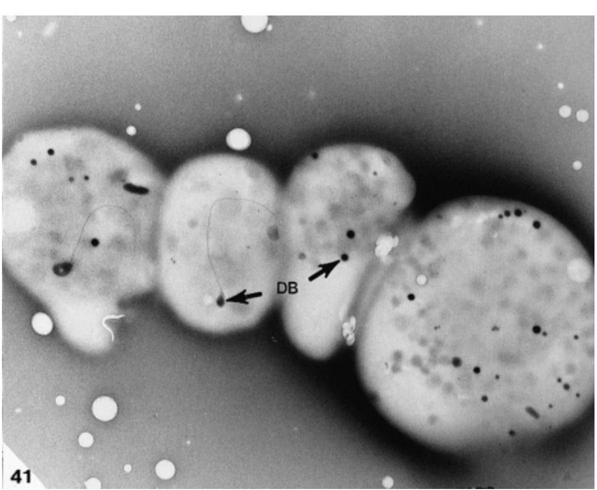


Fig. 3.41. Human platelets examined unfixed and unstained in the transmission electron microscope. Dense bodies (DB) inside the cytoplasm are inherently electron opaque, permitting visualization and enumeration by this technique (Mag ×10 000).

Dense bodies

The dense bodies of blood platelets have been a subject of particular interest. Electron microscopy, ultrastructural autoradiography, analytical electron microscopy, and biochemistry have demonstrated that the inherently electron-opaque dense bodies are the storage sites for the nonmetabolic pool of adenine nucleotides, serotonin, and calcium secreted by human platelets during the release reaction[92,99–103] (Figs. 3.41, 3.42). Dense bodies are virtually absent in platelets from patients with storage pool disease and the Hermansky–Pudlak syndrome[104]. Their absence is associated with defective aggregation in vitro and mild hemorrhagic symptoms in vivo. Richards and Da Prada[105] have introduced a cytochemical technique for localizing adenine nucleotides in organelles storing biogenic amines and have used this method to stain selectively the membranes surrounding platelet dense bodies. Platelets from animals and humans with storage pool disease and patients with Hermansky–Pudlak syndrome were completely negative for the uranaffin reaction. Thus, morphology and ultrastructural cytochemistry have played important roles in characterization of dense bodies, and in diagnosis of patients with platelet storage pool deficiency.

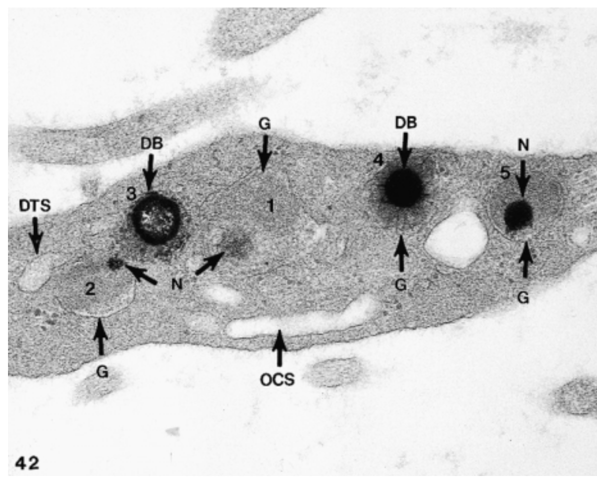


Fig. 3.42. Conversion of platelet granules to dense bodies. The precise origin of platelet dense bodies is still uncertain. In the past we have suggested that they originate from a population of α-granules containing nucleoids. This illustration demonstrates several stages in the development of dense bodies from granules. One granule (1) has a slightly dense nucleoid while the granule (2) next to it has a smaller but more electron-dense spot in its matrix. The nucleoid in a third granule (3) is larger and very dense. Without granule matrix it would be considered to be a dense body. The fourth (4) and fifth (5) organelles are typical dense bodies, but retain granule matrix. Although we no longer believe dense bodies originate from alpha granules, the origin of the dense nucleoids in granules remains uncertain (Mag × 40 000).

Alpha (α) granules

Alpha granules are the most numerous of the several types of organelles in platelet cytoplasm (Figs. 3.2, 3.4, 3.38). Despite relatively similar shape, size and internal matrix appearance, variations are common. Giant platelet α granules are frequent in platelets maintained at room temperature or 37 °C for over 24 hours. They result from fusion of normal sized α granules[106]. The giant granules are noted in

Cambridge Books Online © Cambridge University Press, 2009

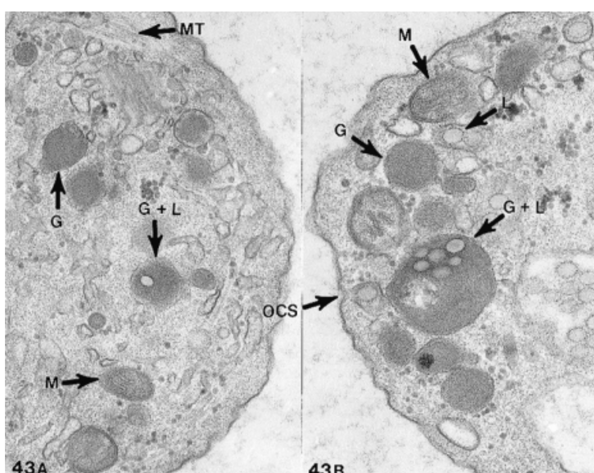


Fig. 3.43(*a*) and (*b*). Platelets from samples of C-PRP exposed to latex for 60 and 90 min, respectively, before fixation. The cells are still relatively discoid with circumferential microtubule (MT) and randomly dispersed organelles, including granules (G) and mitochondria (M). Some of the latex spherules (L) have been transferred from OCS channels to apparently intact granules (G and L) (Mag ×35 000).

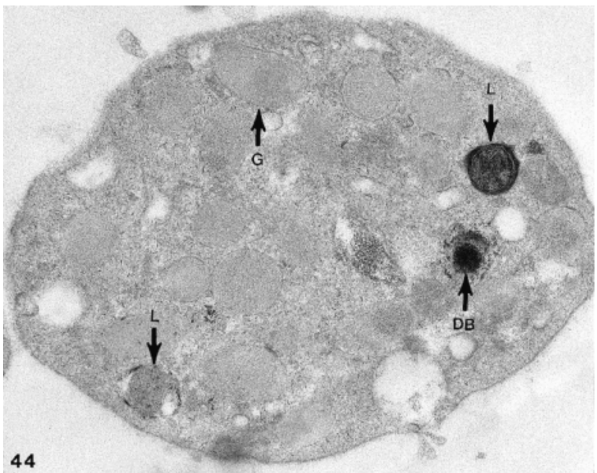


Fig. 3.44. Platelet from a sample of citrated PRP incubated in special medium to demonstrate acid phosphatase. Most granules (G) are devoid of reaction. One organelle is heavily stained by lead salt and another is lightly stained. Because they contain reaction product of acid phosphatase, both organelles are considered lysosomes (L). A dense body (DB) is also present in this cell (Mag ×43 000).

stored platelet samples and represent the earliest morphologically visible storage lesion in the cell[107]. Cross-sections reveal nucleoids as cores of alpha granule matrices that vary in electron opacity. Many nucleoids are as electron opaque as dense bodies and this observation gave rise to the speculation that dense bodies derived from alpha granules[103] (Fig. 3.42). This did not prove to be the case. Cross-sections also revealed microtubule-like structures at the peripheral margin of the matrix in some alpha granules[108], but not in all. Immunohistochemical studies have shown that the tubules are von Willebrand factor[109]. Alpha granules are the storage site for a long list of proteins that facilitate activation, aggregation, hemostasis, clot retraction and repair of the injured blood vessel. Behnke[110] proposed that the various proteins were taken up by coated vesicles formed at the platelet surface membrane and transferred across platelet cytoplasm to alpha granules. As he suggested, platelets were constantly 'sipping the soup'. However, the number of coated vesicles forming at the surface or present in the cytoplasm of resting and activated platelets is so small that the mechanism as a source for alpha granule proteins seems remote. The surface-connected open canalicular system may be a more likely route for uptake and transfer of proteins to alpha granules. It has been shown that small latex spherules (Fig. 3.43), colloidal gold particles and thorium dioxide can be taken up and transferred by the OCS to apparently intact alpha granules without causing platelet activation.[111] Recent studies have shown that platelet alpha granule membranes mirror the platelet plasma membrane in their content of glycoproteins GPIIb–IIIa, CD36, CD9, PECAM-1, Rap 1b, GPIb, GPIX and GPV[112]. This finding may reflect a constant interaction between alpha granules and membranes of the open canalicular system in platelets leading to uptake of proteins.

Lysosomes

Lysosomal organelles are relatively infrequent in human platelets. They have no distinguishing morphological features to separate them from alpha granules. Thus, they require cytochemical techniques to identify them (Fig. 3.44). Platelet lysosomes contain a full complement of hydrolytic enzymes similar to those found in the neutrophil lysosomes. One of them, acid phosphatase, is readily identified by ultrastructural cytochemistry. The only disorder in which platelet lysosomes are involved is the Chediak–Higashi syndrome (CHS)[113,114]. The lysosomes are often enlarged and irregular in form in platelets obtained from patients with CHS. It is uncertain what functions lysosomes serve in normal platelet physiology.

Internal membrane system

Surface-connected open canalicular system (OCS)

The introduction of aldehyde fixation techniques in the early 1960s resulted in markedly improved preservation of platelet ultrastructure and stabilization of the associations

Cambridge Books Online © Cambridge University Press, 2009

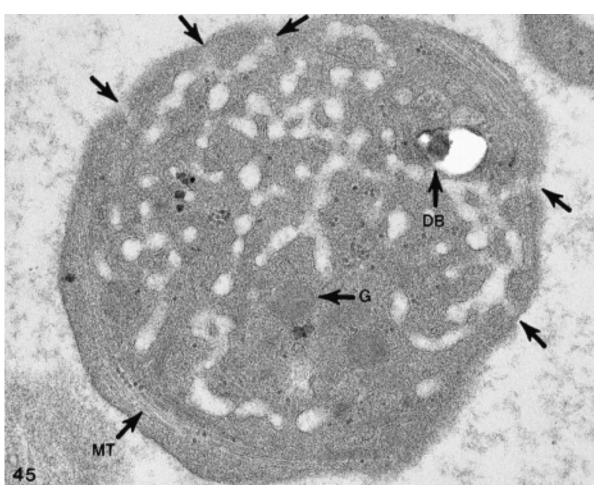


Fig. 3.45. Membrane systems. Discoid platelet sectioned near the horizontal plane. Portions of the circumferential band of microtubules (MT) are apparent near the cell periphery. Dense bodies (DB), granules (G) and other cytoplasmic constituents are spread randomly in the cell substances. Some of the larger black particles in the background are due to osmium precipitate. The tortuous nature of the OCS is apparent in this example. At least five openings (*arrows*) onto the cell surface can be identified. The alternating dilatations and constrictions in the channels produce a tortuous appearance. Channels branch shortly after entering the cell and communicate with each other. The result is a spider web-like maze of interconnecting channels that spreads throughout the cytoplasm and communicates directly with the cell surface at several sites (Mag ×43 000).

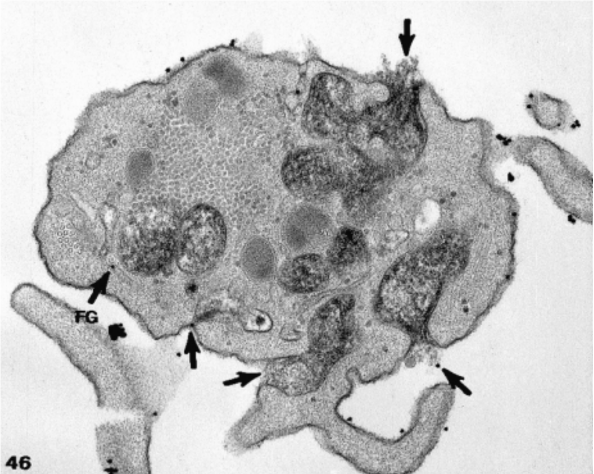


Fig. 3.46. Platelet fixed 15 seconds after exposure to 0.2 U/ml of thrombin in the presence of fibrinogen-coated gold particles. Shape change is limited, but many granules are tannic acid positive. Many have fused with channels of the open canalicular system (↑) and are in the process of discharge to the exterior. Fibrinogen-gold (FG) particles cover the exposed surface and have entered the OCS (Mag ×36 000).

between the surface membrane and what had been regarded as vesicles and vacuoles in early studies[115]. The OCS revealed by improved fixation consisted of tortuous invaginations of the cell wall tunneling throughout the cytoplasm in a serpentine fashion (Fig. 3.5, 3.45). Studies employing electron-dense tracers revealed that channels of the OCS were patent in activated and aggregated platelets as well as in unaltered cells[12]. Vesicles and vacuoles were virtually absent in doubly fixed platelets, suggesting that these elements were in reality components of the OCS. It was clear from these investigations that canaliculi of the OCS greatly increased the total surface area of the platelet exposed to plasma and provided a route for chemical or particulate substances to reach the deepest recesses of the cell. The observation that the OCS remained patent throughout the processes of platelet shape change, internal transformation, contraction, adhesion, and aggregation suggested that the channels might serve as conduits for substances extruded by platelets during the release reaction[116,117] (Figs. 3.46, 3.47). Experiments employing the cationic polyelectrolytes polylysine and polybrene dem-

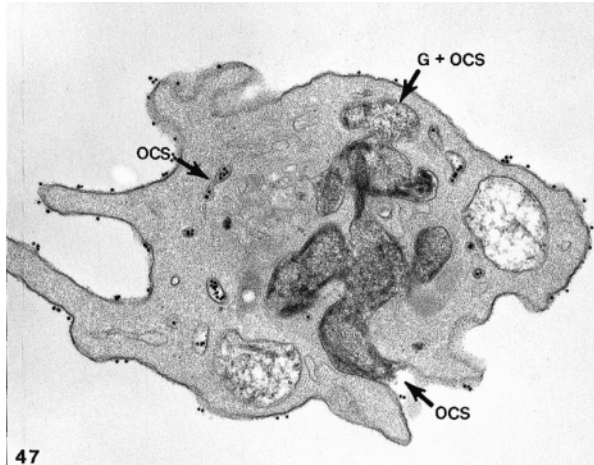


Fig. 3.47. Platelet exposed to fibrinogen gold and then to 0.2 U/ml of thrombin for 30 seconds before fixation and staining with tannic acid. Shape change and internal transformation are reasonably advanced and many tannic acid positive alpha granules are fused to OCS channels (G + OCS) delivering their contents to the exterior. Fibrinogen coated gold particles have penetrated many OCS channels (Mag ×36 000).

Cambridge Books Online © Cambridge University Press, 2009

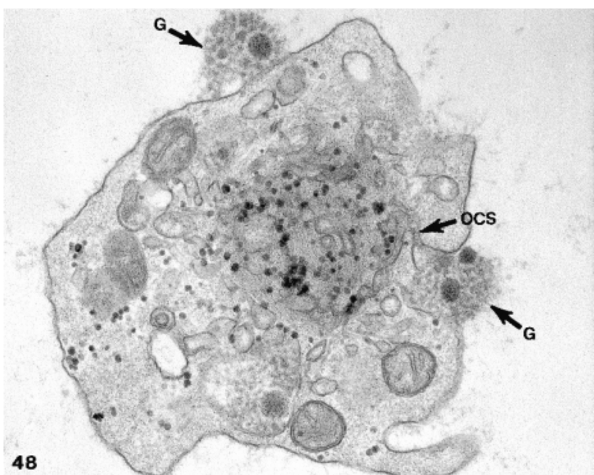


Fig. 3.48. Platelet from washed suspension combined with polylysine and then exposed to thrombin (0.5 U/ml) for 30 seconds before fixation. Polylysine has been taken up by alpha granules (G), some of which are connected to the OCS and in the process of discharging their contents to the exterior. The extruded granules remain intact outside the platelet, despite loss of their enclosing membranes (Mag ×36 000).

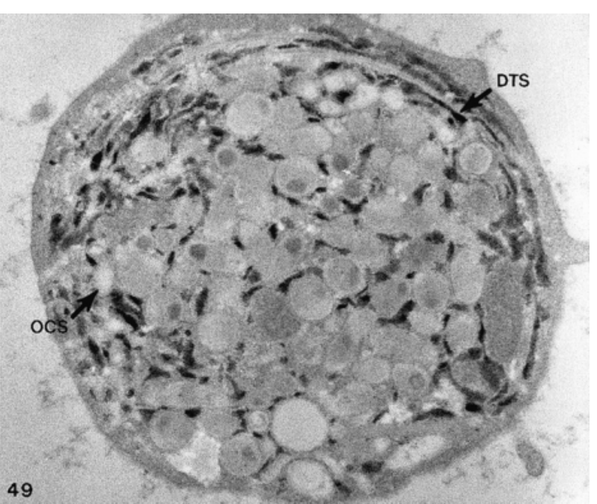


Fig. 3.49. Cytochemistry of membrane systems. This platelet is from a sample of PRP incubated for peroxidase activity. Enzyme reaction product is specifically localized to channels of the DTS and none is present in the surface-connected OCS (Mag × 31 000).

onstrated that this supposition was correct[118,119] (Fig. 3.48). Recent studies have shown that channels of the OCS remain open during clot retraction and continue to serve as the egress route for products of dense bodies and alpha granules[120].

Dense tubular system (DTS)

Channels of the DTS were distinguished from clear canaliculi of the OCS by an amorphous material similar in opacity to surrounding cytoplasm concentrated within them that stains for peroxidase (Fig. 3.49) and binds lead (Fig. 3.50)[115]. Like the OCS, channels of the DTS were randomly dispersed in the platelet cytoplasm. In addition, a channel or two of the OCS could be identified in close association with the circumferential band of microtubules in most thin sections of platelets (Figs. 3.2, 3.4). Investigations of this relationship suggested that the DTS might have an important role in the elaboration and stabilization of the circumferential microtubules supporting the discoid form of platelets. Additional studies demonstrated that the DTS originated from rough endoplasmic reticulum in the parent megakaryocyte and was, therefore, residual smooth endoplasmic reticulum[18]. There was no evidence of a physical communication between channels of the OCS and DTS, although such a communication had been suggested by some workers. Thus, platelets have two discrete membrane systems not found in other blood cells,

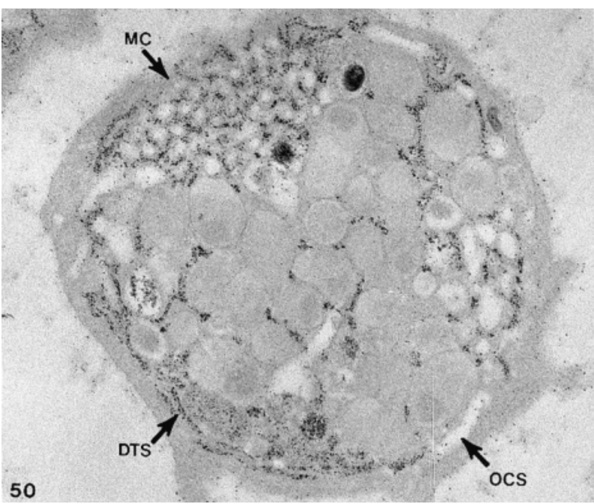


Fig. 3.50. Cytochemistry of membrane systems. The cell is from a sample of glutaraldehyde-fixed platelets incubated in a solution containing lead ions. Lead has selectively deposited in channels of the DTS. None is present in the surface-connected OCS. Note the proximity of lead-filled channels to open channels in the membrane complex (MC). The same procedure has been used to demonstrate calcium-binding sites in the sarcoplasmic reticulum of muscle (Mag ×38 000).

Cambridge Books Online © Cambridge University Press, 2009

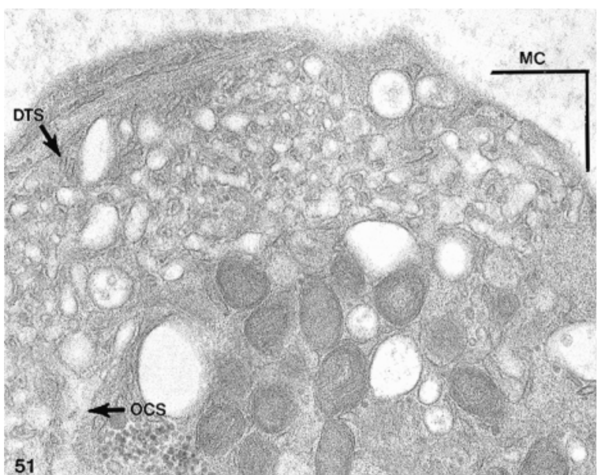


Fig. 3.51. Membrane systems. The sponge-like appearance of the peripheral cytoplasm in this cell is due to the interwoven associations between elements of the surface-connected OCS and the DTS. The resulting membrane complex (MC) strongly resembles the associations between transverse tubules and sarcotubules in muscle cells (Mag ×46 000).

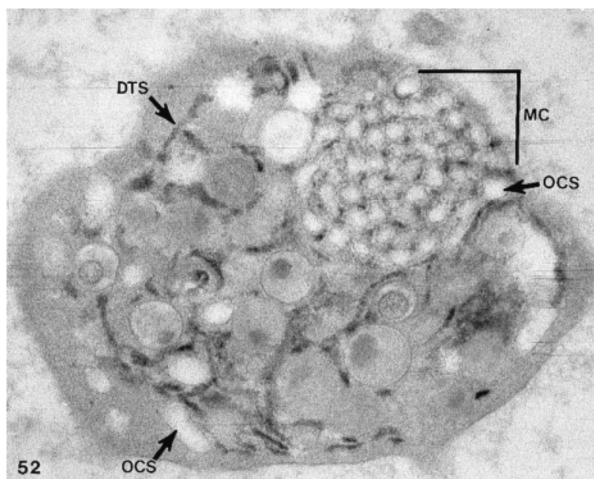


Fig. 3.52. Cytochemistry of membrane systems. The platelet is from a sample reacted for endogenous peroxidase activity. Reaction product is localized to channels of the DTS. Clear channels of the surface-connected OCS form very close associations with the DTS in membrane complexes (MC) (Mag × 48 000).

the OCS derived from the plasma membrane of the megakaryocyte and the DTS representing residual smooth endoplasmic reticulum of the parent cell.

Membrane complexes

The OCS and DTS are not completely isolated membrane systems. Close inspection of thin sections of well-preserved platelets revealed that canaliculi of the OCS and DTS form intimate physical relationships in nearly every cell (Figs. 3.50–3.52). The association of the two channel systems was usually restricted to one or two areas of the cytoplasm, and in most examples these areas were eccentrically located. Elements of the OCS in such areas were gathered in clusters or groups. Even though the open channels were closely approximated, small canaliculi of the DTS could be identified interspersed between them. The relationship was particularly prominent in platelets stained for peroxidase activity in which dense reaction product delineated channels of the DTS and outlined their extremely close relationship to cluster of open canaliculi[121] (Fig. 3.52). Examination of the membrane complexes at higher magnification revealed that elements of the DTS were the only structures interspersed between open canaliculi, and that membranes of the two channel systems were practically in apposition. Replicas of freeze–fractured platelets revealed an identical arrangement of the two channel systems in complexes[122]. When studied by this method, the OCS is clearly seen to be a fenestrated membrane system and channels of the DTS are located in each window or fenestra.

Electron-microscopic studies that defined the platelet as a form of muscle cell were crucial in identifying and characterizing the membrane systems involved in regulating activation of the contractile system. Contraction of platelet actomyosin, as in other muscle systems, is modulated by calcium flux. Calcium is maintained at low levels in the cell until the stimulus for contraction is received at the surface or sarcolemma. In skeletal muscle a system of channels, derived from the cell wall and referred to as the transverse tubular system, conducts the signal to the cell interior[123]. Transmission from the transverse tubular system is, in turn, mediated by a second group of channels called the sarcotubular system derived from endoplasmic reticulum in the embryonic muscle cell. The sarcotubules contain higher concentrations of calcium than the cytoplasm. Release of calcium from the sarcotubules to the cytoplasm triggers activation of the contractile elements, and relaxation develops when calcium is pumped back into the sarcotubules.

The similarity of the platelet membrane systems and membrane complexes to the transverse tubules and sarcotubules of muscle led to a series of investigations comparing them[122]. These studies have shown that the DTS is the calcium-sequestering site in platelets (Fig. 3.50), resembling the sarcotubules of muscle cells, and that the channels of the OCS are similar to the transverse tubules of muscle. Furthermore, the membrane complexes closely

Cambridge Books Online © Cambridge University Press, 2009

resemble the diads and triads formed between the transverse tubules and sarcotubules in muscle cells (Fig. 3.51). The proximity of the dual channel system in platelets to the contractile elements, and the demonstration that the DTS selectively binds divalent cations, that platelets do not require exterior calcium for activation, and that vesicles from platelet membranes can sequester calcium just as well as sarcotubular vesicles from muscle, strongly support the concept that the OCS and DTS in platelets are the equivalent of the sarcoplasmic reticulum of muscle cells[122] (Fig. 3.52).

In 1963, Grette[124] showed that pig platelets contained a relaxing factor that could prevent clot retraction presumably by sequestering calcium. This factor was identified as a membrane vesicle by Statland et al.[125] in 1969 and has been characterized by a number of groups since then[126]. Additional evidence has localized the Ca^{2+}/Mg^{2+} ATPase to the DTS, although some activity may be in the channels of the surface-connected canalicular system[127]. Thus, platelets appear to be able to pump calcium out of the cytoplasm into the DTS and perhaps also outside the platelet. The activity of this calcium pump is essential to maintaining a low cytoplasmic calcium concentration, which may in turn keep the microtubules polymerized and the cell in its resting discoid state[128]. The activity of the calcium pump is enhanced by the presence of intracellular cyclic adenosine 3′5′-monophosphate (cAMP), which is important as an inhibitor of platelet function[129]. Many agents that inhibit platelet function, including adenosine, prostaglandin E_1, prostaglandin D_2 and prostaglandin I_2, act by stimulating the platelet adenylate cyclases to cause a rise in intracellular cAMP. The mechanism whereby cAMP enhances the calcium pump activity is unknown. However, cAMP may well work through a protein kinase and phosphorylation of a protein associated with the calcium pump[130].

An interest in prostaglandin synthesis developed from investigations of platelets from patients with the Hermansky–Pudlak syndrome (HPS), the classic form of storage pool deficiency[131]. HPS platelets are deficient in dense bodies, serotonin, and adenine nucleotides and fail to undergo the second wave of aggregation when stimulated by agents that cause the release reaction. Normal platelets treated with aspirin also fail to develop second waves of aggregation when exposed to these agents because aspirin blocks prostaglandin synthesis. The similar defects in the response of aspirin-treated and HPS platelets to aggregating agents led us to carry out an unusual experiment. We combined equal volumes of HPS and aspirin-treated normal platelets and exposed samples to aggregating agents. The results demonstrated that the mixed population of aspirin-treated normal cells and HPS platelets responded as well as untreated normal control platelets to all aggregating agents.[132] In subsequent investigations, we demonstrated that an aspirin-sensitive factor in HPS platelets stimulated by exposure to the aggregating agent overcame the influence of aspirin on the normal cells, resulting in the release of their storage pool and normalization of the second wave of aggregation[133]. This was the initial demonstration of intercellular communication between platelets and revealed for the first time that two abnormal populations of platelets could compensate for each other's defect.

Results of this study led to investigation of platelets by combined biochemical and ultrastructural techniques for the site of prostaglandin synthesis. Ultrastructural cytochemical studies by Janine Breton-Gorius[134] and by us[18] had demonstrated that platelets contain a specific peroxidase activity localized to channels of the DTS. It is our practice to test agents that inhibit cytochemical reactions on living cell systems. As a result, we examined the effect of 3-amino-1,2,4-triazole (AMT),an inhibitor of platelet peroxidase, on platelet aggregation[135]. The effects of the agent were similar to those of aspirin. Concentrations of AMT that blocked cytochemical demonstration of peroxidase inhibited the second wave of aggregation stimulated by release-inducing aggregating agents. Subsequent studies revealed that AMT blocked prostaglandin formation and suggested that the peroxidase was involved in the synthetic process. Other workers have shown that peroxidase activity is contained on the prostaglandin endoperoxide synthetase enzyme during purification of the enzyme and that the heme groups of cyclooxygenase are essential for its prostaglandin synthetase activity. These findings and studies on platelet subcellular fractions showing that prostaglandin synthetic activity separates with endoplasmic reticulum enzymes and not plasma membrane enzymes[136] led to the conclusion that the DTS is the site of platelet prostaglandin synthesis[137].

In summary, morphology and ultrastructure have provided the palette on which to mix details of platelet biochemistry, physiology and pathology. Remaining details will soon be added to the final picture and, in essence, our work will be complete.

References

1 Clay RS, Court TH. *The history of the microscope*. London: Charles Griffin & Co., 1932; pp 20–436.

2 Leeuwenhoek A van. Microscopical observations. *Philos Trans R Soc Lond* 1674; pp 121–128.

Cambridge Books Online © Cambridge University Press, 2009

3 Hewson W. *Experimental inquiries: Part 1. Containing an inquiry into the properties of the blood*, 2nd ed., London: J Johnson, 1774.
4 Donné A. De l-origine des globules du sang, de leur mode de formation et de leur fin. *C R Seances Acad Sci* 1842; 14: 366–368.
5 Bizzozero J. Ueber einen neuen formbestandheil des bleetes und dessen rolle bei der thrombose und der blutgerinnung. *Arch Path Anat Phipiol* 1882; 90: 261–332.
6 Tocantins LM. The mammalian blood platelet in health and disease. *Medicine* 1938; 17: 155–260.
7 Roth GJ. Developing relationships: Arterial platelet adhesion, glycoprotein Ib, and leucine-rich glycoproteins. *Blood* 1991; 77: 5–19.
8 Saelman EUM, Nieuwenhuis HK, Hese KM et al. Platelet adhesion to collagen types I through VIII under conditions of stasis and flow is mediated by GPIa/IIa ($\alpha_2\beta_1$-integrin). *Blood* 1994; 83: 1244–1250.
9 Sixma JJ, Wester J. The haemostatic plug. *Sem Hematol* 1977; 14: 265–299.
10 Sixma JJ, van Zanten GH, Banga JD, Nieuwenhuis HK, DeGroot PG. Platelet adhesion. *Sem Hematol* 1995; 32: 1–6.
11 White JG. Platelet structural physiology: The ultrastructure of adhesion, secretion and aggregation in arterial thrombosis. In *Thrombosis and platelets in myocardial ischemia. cardiovascular clinics* 1987; 18: 13–33.
12 White JG. Platelet morphology. In Johnson, SA, ed. *The circulating platelet.* New York: Academic Press, 1971; pp 45–121.
13 White JG. The ultrastructural cytochemistry and physiology of blood platelets. In Mostafi FK, Brinkhous KM, eds. *The platelet.* Baltimore: Williams and Wilkins; 1971: pp. 83–115.
14 Clemetson KJ. Platelet activation: Signal transduction via membrane receptors. *Thromb Haemost* 1995; 74: 111–116.
15 Nurden AT, Caen JP. Specific roles for platelet surface glycoproteins in platelet function. *Nature* 1975; 255: 720–722.
16 Shattil SJ, Bennett JS. Platelets and their membranes in hemostasis: physiology and pathology. *Ann Intern Med* 1981; 94: 108–118.
17 White JG, Gerrard JM. The cell biology of platelets. In Weissman G, ed. *Handbook of inflammation* (*The cell biology of inflammation*), Elsevier/North Holland, Inc., New York; 1980: 83–143.
18 White JG. Interaction of membrane systems in blood platelets. *Am J Pathol* 1972; 66: 295–312.
19 Hartwig JH, Barkalow K, Anser A, Italliano J. The elegant platelet: Signal controlling actin assembly. *Thromb Haemost* 1999; 82: 392–398.
20 Roskam J. Contribution a l'etude de la physiologie normale et pathologique du globulin. *Arch Int Physiol* 1923; 20: 241–257.
21 Adelosn E, Rheingold JJ, Crosby WH. The platelet as a sponge: a review. *Blood* 1961; 17: 767–785.
22 Weiss HJ: Flow related platelet deposition on subendothelium. *Thromb Haemost* 1995; 74: 117–122.
23 White JG. Transfer of thorium particles from plasma to platelets and platelet granules. *Am J Pathol* 1968; 53: 567–575.
24 White JG, Clawson CC. Effects of large latex particle uptake on the surface connected canalicular system of blood platelets: a freeze–fracture and cytochemical study. *Ultrastruct Path* 1981; 2: 277–287.
25 White JG. An overview of platelet structural physiology. *Scan Micr* 1988; 1: 1677–1700.
26 White JG, Krumweide M. Low voltage, high resolution scanning electron microscopy (SEM) of resting and surface activated platelets. *Blood* 1990; 76: 481A.
27 White JG. The secretory pathway of bovine platelets. *Blood* 1987; 69: 878–885.
28 Hartwig JH. Mechanisms of actin rearrangements mediating platelet activation. *J Cell Biol* 1992; 118: 1421–1442.
29 White JG. Platelet microtubules and microfilaments: effects of cytochalasin B on structure and function. In Caen J, ed. *Platelet aggregation.* Paris: Masson & Cie; 1971: 15–52.
30 Bessin M, Weed RI, Leblond PF, eds. *Red cell shape.* New York: Springer-Verlag; 1973: 180.
31 Escolar G, Leistikow E, White JG. The fate of the open canalicular system in surface and suspension-activated platelets. *Blood* 1989; 74: 1983–1988.
32 Frojmovic MM, Wong T, White JG. Platelet plasma membrane is equally distributed between surface and osmotically-evaginable surface-connecting membrane, independent of size, subpopulation or species. *Nouv Rev Fr Hematol* 1992; 34: 99–110.
33 Zucker-Franklin D, Benson KA, Myers KM. Absence of a surface-connected canalicular system in bovine platelets. *Blood* 1985; 65: 241–244.
34 Grouse LH, Rao GHR, Weiss DJ, Perman V, White JG. Surface-activated bovine platelets do not spread, they unfold. *Am J Pathol* 1990; 136: 399–408.
35 Sims PJ, Faioni EM, Wiedmer T, Shattil SJ. Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity. *J Biol Chem* 1988; 263: 18205–18212.
36 Heijen HF, Sahiel AE, Fijnheer R, Geuze HJ and Sixma JJ. Activated platelets release two types of membrane vesicles: microvesicles by surface shredding and exosomes derived from exocytosis of multivesicular bodies and α-granules. *Blood* 1999; 94: 3791–3799.
37 Clemetson KJ. Glycoproteins of the platelet plasma membrane. In George JN, Nurden AT, Phillips DR, eds. *Platelet Membrane Glycoproteins.* New York: Plenum Press, 1985, 51–85.
38 Phillips DR. Receptors for platelet agonists. In George JN, Nurden AT, Phillips DR, eds. *Platelet Membrane Glycoproteins.* New York: Plenum Press; 1985: 145–169.
39 Kunicki TJ. Platelet glycoprotein antigens and immune receptors. In Jamieson GA, ed. *Platelet membrane receptors: molecular biology, immunology, biochemistry and pathology. Prog Clin Biol Res* 1988; 283: 87–125.

Cambridge Books Online © Cambridge University Press, 2009

40 Bourguignon LYW. Receptor capping in platelet membranes. *Cell Biol Int Rep* 1984; 8: 19–26.

41 Santoso S, Kiefel V, Mueller-Eckhardt C. Redistribution of platelet glycoproteins induced by allo- and auto-antibodies. *Thromb Haemost* 1987; 58: 866–871.

42 Kakaiya RM, Kiraly TL, Cable RG. Concavalin A induces patching/capping of the platelet membrane glycoprotein IIb-IIIa complex. *Thromb Haemost* 1988; 59: 281–283.

43 Polley MJ, Leung LLK, Clark FY, Nachmias RL. Thrombin-induced membrane glycoprotein IIb and IIIa complex formation. An electron microscope study. *J Exp Med* 1981; 154: 1058–1068.

44 Isenberg WM, McEver RP, Phillips DR, Shuman MA, Bainton DF. The platelet fibrinogen receptor: An immunogold-surface replica study of agonist induced ligand binding and receptor clustering. *J Cell Biol* 1987; 104: 1655–1663.

45 Santoso S, Zimmerman U, Neppert J, Mueller-Eckhardt C. Receptor patching and capping of platelet membranes induced by monoclonal antibodies. *Blood* 1986; 67: 343–349.

46 Albrecht RM, Goodman SL, Simmons SR. Distribution and movement of membrane associated platelet glycoproteins: Use of colloidal gold with correlative video-enhanced light microscopy, low voltage, high resolution scanning electron microscopy and high voltage transmission electron microscopy. *Am J Anat* 1989; 185: 149–164.

47 Loftus JC, Albrecht RM. Redistribution of the fibrinogen receptor of human platelets after surface activation. *J Cell Biol* 1984; 99: 822–829.

48 Goodman SL, Albrecht RM. Correlative light and electron microscopy of platelet adhesion and fibrinogen receptor expression using colloidal-gold labeling. *Scand Micr* 1987; 1: 727–734.

49 Oliver JA, Albrecht RM. Colloidal gold labeling of fibrinogen receptors in epinephrine and ADP-activated platelet suspensions. *Scan Micr* 1987: 1: 745–756.

50 Hourdille P, Heilmann R, Combrie R, Winckler J, Clemetson KJ, Nurden AT. Thrombin induces a rapid redistribution of glycoprotein Ib–IX complexes within the membrane systems of activated platelets. *Blood* 1990; 76: 1503–1513.

51 Hourdille P, Bralnick HR, Heilmann E, Derlon A, Ferrer AM, Vezon G, Nurden AT. von Willebrand factor bound to glycoprotein Ib is cleared from the platelet surface after platelet activation by thrombin. *Blood* 1992; 79: 2011–2021.

52 Kieffer N, Guichard J, Breton-Gorius J. Dynamic redistribution of major platelet surface receptors after contact-induced platelet activation and spreading: An immunoelectron microscopy study. *Am J Pathol* 1992; 140: 57–73.

53 Leistikow EA, Barnhart MI, Albrecht RM, White JG. Redistribution of fibrinogen receptors on surface-activated platelets. In Mossesson MH, ed. Fibrinogen 3. *Biochemistry, biological functions, gene regulation and expression.* New York: Elsevier Science Publishers, 1988: 215–220.

54 Leistikow EA, Barnhart MI, White JG. Receptor-ligand complexes are cleared to the open canalicular system of surface-activated platelets. *Br J Haematol* 1990; 74: 93–100.

55 White JG, Escolar G. Induction of receptor clustering, patching and capping on surface-activated platelets. *Lab Invest* 1990; 63: 332–340.

56 White JG. Separate and combined interactions of fibrinogen-gold and latex with surface-activated platelets. *Am J Pathol* 1990; 137: 989–998.

57 Escolar G, Leistikow E, White JG. The fate of the open canalicular system in surface and suspension-activated platelets. *Blood* 1989; 74: 1983–1988.

58 Escolar G, White JG. The platelet open canalicular system: a final common pathway. *Blood Cells* 1991; 17: 467–485.

59 White JG, Escolar G. The blood platelet open canalicular system: A two-way street. *Eur J Cell Biol* 1991; 56: 233–242.

60 Escolar G, Clemetson K, White JG. Persistence of mobile receptors on surface- and suspension-activated platelets. *J Lab Clin Med* 1994; 123: 536–546.

61 White JG. Functional significance of mobile receptors on human platelets. *Arteriosclerosis Thromb* 1993; 13: 1236–1243.

62 White JG, Krumwiede MD, Cocking-Johnson D, Escolar G. Redistribution of GPIb/IX and GPIIb/IIIa during spreading of discoid platelets. *Br J Haematol* 1995; 90: 630–644.

63 White JG, Krumwiede MD, Cocking-Johnson DJ, Escolar G. Dynamic redistribution of glycoprotein Ib/IX on surface-activated platelets: a second look. *Am J Pathol* 1995; 147: 1057–1067.

64 White JG, Krumwiede MD, Cocking-Johnson D, Escolar G. Influence of combined thrombin stimulation, surface activation, and receptor occupancy on organization of GPIb/IX receptors on human platelets. *Br J Haematol* 1994; 88: 137–148.

65 White JG, Conard WJ. The fine structure of freeze fractured platelets. *Am J Pathol* 1973; 70: 45–56.

66 Schick PK, Kurica KB, Chacko GK. Location of phosphatidylethanolamine and phosphatidylserine in human platelet plasma membrane. *J Clin Invest* 1976; 57: 1221–1233.

67 Fantl P, Ward HA. The thromboplastic component of intact blood platelets is present in masked form. *Aust J Exp Biol Med Sci* 1955; 36: 499–507.

68 Norne WC, Norman NE, Schwartz DB, Simons ER. Changes in cytoplasmic pH and in membrane potential in thrombin stimulated platelets. *Eur J Biochem* 1981; 120: 295–302.

69 White JG. The submembrane filaments of blood platelets. *Am J Pathol* 1969; 56: 267–277.

70 Cohen I, Gerrard JM, Bergman RN, White JG. The role of contractile microfilaments in platelet activation. In: Peeters H, ed. *Protides of the Biological Fluids.* Oxford: Pergamon Press; 1979: 555–566.

71 David-Ferreira JF. The blood platelet: electron microscopic studies. *Int Rev Cytol* 1964; 55: 89–103.

72 Haydon GB, Taylor AB. Microtubules in hamster platelets. *J Cell Biol* 1965; 26: 673–675.

73 Behnke O. Further studies on microtubules: a marginal bundle in human and rat thrombocytes. *J Ultrastruct Res* 1965; 13: 469–477.

Cambridge Books Online © Cambridge University Press, 2009

74 Bessis M, Breton-Gorius J. Les microtubules et les fibrilles dan les plaquettes etalies. *Nouv Rev Fr Hematol* 1965; 5: 657–662.
75 Pipeleers DG, Pipeleers-Marichal MA, Kipnis DM. Physiological regulation of total tubulin and polymerized tubulin synthesis. *J Cell Biol* 1977; 74: 351–357.
76 White JG. The substructure of human platelet microtubules. *Blood* 1968; 32: 638–648.
77 Zucker MB, Borrelli J. Reversible alterations in platelet morphology produced by anticoagulants and by cold. *Blood* 1954; 9: 602–608.
78 White JG, Krivit W. An ultrastructural basis for the shape changes induced in platelets by chilling. *Blood* 1967; 30: 625–635.
79 White JG. Effects of colchicines and vinca alkaloids on human platelets. I. Influence on platelet microtubules and contractile function. *Am J Pathol* 1968; 53: 281–291.
80 White JG. Effects of ethylenediamine tetracetic acid (EDTA) on platelet structure. *Scand J Haematol* 1968; 5: 241–250.
81 White JG, Krumwiede MD, Escolar G. EDTA induced changes in platelet structure and function: Influence on particle uptake. *Platelets* 1999; 10: 327–337.
82 Takiuchi K, Kuroda K, Ishigami M, Nakamura T. Actin cytoskeleton of resting bovine platelets. *Exp Cell Res* 1990; 186: 374–380.
83 Smith CM II, Burris SM, Weiss DJ, White JG. Comparison of bovine and human platelet deformability using micropipette elastimetry. *Am J Vet Res* 1989; 50: 34–38.
84 Grouse LH, Rao GHR, Weiss DJ, Perman V, White JG. Surface-activated bovine platelets do not spread, they unfold. *Am J Pathol* 1990; 136: 339–408.
85 Hartwig JH, DeSisto M. The cytoskeleton of the resting human blood platelet: structure of the membrane skeleton and its attachment to actin filaments. *J Cell Biol* 1991; 112: 407–425.
86 Hartwig JH. Mechanism of actin rearrangements mediating platelet activation. *J Cell Biol* 1992; 118: 1421–1442.
87 Winokur R, Hartwig J. Mechanism of shape change in chilled human platelets. *Blood* 1995; 85: 1796–1804.
88 White JG, Rao GHR. Microtubule coils versus the surface membrane cytoskeleton in maintenance and restoration of platelet discoid shape. *Am J Pathol* 1998; 165: 597–610.
89 White JG, de Alarcon P. Platelet spherocytosis: A new bleeding disorder. *Am J Hematol*, in press.
90 Gerrard JM, White JG. The structure and function of platelets, with emphasis on their contractile nature. In: Vol. 6. Iochim HL, ed. *Pathobiology Annual*, New York: Appleton-Century-Crofts; 1976: 31–58.
91 Hayem G. Sur le mechanism de l'arrêt des hemorrhagies. *C.R.Hebd. Séanc. Acad Sci, Paris* 1882; 95: 18–36.
92 Muller HF. Übereinen bisher nicht beachteten Formbestandtheil des Blutes. *Zentbl. Allg. Path. Anat* 1896; 7: 529.
93 Wood JG. Electron microscopic localization of 5-hydroxytryptamine (5-HT). *Texas Rep Biol Med* 1965; 23: 828–837.
94 Breton-Gorius J, Guichard J. Two different types of granules in megakaryocytes and platelets as revealed by the diaminobenzidine reaction. *J Micros Biol* 1975; 23: 197–202.
95 Bentfield ME, Bainton DF. Cytochemical localization of lysosomal enzymes in rat megakaryocytes and platelets. *J Clin Invest* 1975; 56: 1635–1639.
96 Da Prada M, Jakabova R, Luscher EF, Pletscher A, Richards JG. Subcellular localization of the heparin-neutralizing factor in blood platelets. *J Physiol* 1976; 257: 495–501.
97 White JG. Ultrastructural studies of the gray platelet syndrome. *Am J Pathol* 1979; 95: 335–462.
98 Gerrard JM, Phillips DR, Rao GHR et al. Biochemical studies of two patients with the gray platelet syndrome–Selective deficiency of platelet alpha granules. *J Clin Invest* 1980; 66: 102–109.
99 Tranzer JP, Da Prada M, Plescher A. Letter to the editor. Ultrastructural localization of 5-hydroxytryptamine in blood platelets. *Nature* 1966; 212: 1574–1575.
100 Da Prada M, Pletcher A, Tranzer JP, Knuchel H. Subcellular localization of 5-hydroxytryptamine in blood platelets. *Nature* 1967; 216: 1315–1317.
101 Bak KJ, Hassler R, May B, Westerman E. Morphological and biochemical studies on the storage of serotonin and histamine in blood platelets of the rabbit. *Life Sci* 1967; 6: 1133–1146.
102 Davis RB, White JG. Localization of 5-hydroxytryptamine in blood platelets. A radioautographic and ultrastructural study. *Br J Haematol* 1968; 15: 93–99.
103 White JG, Origin and function of platelet dense bodies. *Ser Haematol* 1970; 3: 17–46.
104 White JG, Gerrard JM. Ultrastructural features of abnormal blood platelets. *Am J Pathol* 1976; 83: 590–632.
105 Richards JG, Da Prada M. Uranaffin reaction: a new cytochemical technique for the localization of adenine nucleotides in organelles storing biogenic amines. *J Histochem Cytochem* 1977; 25: 1322–1336.
106 White JG. Platelet membrane interactions. *Platelets* 1999; 10: 368–381.
107 White JG, Clawson CC. Development of giant granules in platelets during prolonged storage. *Am J Pathol* 1980; 101: 635–646.
108 Whit JG. Tubular elements in platelet granules. *Blood* 1968; 32: 148–156.
109 Cramer E, Meyer D, Le Menn R, Breton-Gorius J. Eccentric localization of von Willebrand factor in an internal structure of platelet α-granule resembling that of Weibel–Palade bodies. *Blood* 1985; 66: 710–716.
110 Behnke O. Coated pits and vesicles transfer plasma components to platelet granules. *Thromb Haemost* 1989; 62: 718–722.
111 White JG. Transfer of thorium particles from plasma to platelets and platelet granules. *Am J Pathol* 1968; 53: 567–575.
112 Berger G, Massé JM, Cramer EM. Alpha-granule membrane mirrors the platelet plasma membrane and contains the glycoproteins Ib, IX and V. *Blood* 1996; 87: 1385–1395.

Cambridge Books Online © Cambridge University Press, 2009

113 White JG. Platelet microtubules and giant granules in the Chediak–Higashi syndrome. *Am J Med Technol* 1978; 44: 273–278.
114 Parmely RT, Poon MC, Crist WM, Molluk A. Giant platelet granules in a child with the Chediak-Hagashi syndrome. *Am J Hematol* 1979; 6: 51–60.
115 Behnke O. The morphology of blood platelet membrane systems. *Ser Haematol* 1970; 3: 3–16.
116 White JG. Electron microscopic studies of platelet secretion. *Prog Hemost Thromb* 1974; 2: 49–98.
117 White JG, Krumwiede M. Further studies of the secretory pathway in thrombin-stimulated human platelets. *Blood* 1987; 69: 1196–1203.
118 White JG. Exocytosis of secretory organelles from blood platelets incubated with cationic polypeptides. *Am J Pathol* 1972; 69: 41–49.
119 White JG. The secretory process in platelets. In Cantin M, ed. *The cell biology of the secretory process.* New York, NY: Karger; 1984: 541–546.
120 White JG. Platelet secretion during clot retraction. *Platelets* 2000; 11: 331–343.
121 Breton-Gorius J, Guichard J. Ultrastructural localization of peroxidase activity in human platelets and megakaryocytes. *Am J Pathol* 1972; 66: 277–286.
122 White JG. Is the canalicular system the equivalent of the muscle sarcoplasmic reticulum? *Hemostasis* 1975; 4: 185–191.
123 Taba M, Yamamoto T, Tonomura Y. Molecular mechanism of active calcium transport by sarcoplasmic reticulum. *Physiol Rev* 1978; 58: 1–79.
124 Grette K. Relaxing factor in extracts of blood platelets and its function in the cells. *Nature* 1963; 198: 488–489.
125 Statland BE, Heagan BM, White JG. Uptake of calcium by platelet relaxing factor. *Nature* 1969; 223: 521–522.
126 Robblee LS, Shepro D, Belamarich FA. Calcium uptake and associated adenosine triphosphate activity of isolated platelet membranes. *J Gen Physiol* 1973; 61: 462–481.
127 Cutler L, Rodan G, Feinstein MB. Cytochemical localization of adenylate cyclase and of calcium ion, magnesium ion-activated ATP-ases in the dense tubular system of human blood platelets. *Biochim Biophys Acta* 1978; 542: 357–371.
128 White JG. Ultrastructural lesions of stored platelets. *Vox Sang* 40 (Suppl); 1981: 62–68.
129 Kaser-Glanzmann R, Jakabova M, George JN, Luscher EF. Stimulation of calcium uptake in platelet membrane vesicles by adenosine 3′5′-cyclic monophosphate and protein kinase. *Biochim Biophys Acta* 1977; 466: 429–440.
130 Haslam RJ. Interactions of the pharmacological receptors of blood platelets with adenylate cyclase. *Ser Haematol* 1973; 6: 330–350.
131 Witkop CJ Jr, Hill CW, Desnick SJ, Thies JF, Thorn HL, Jenskins M, White JG. Opthalmologic, biochemical, platelet and ultrastructural defects in various types of oculocutaneous albinism. *J Invest Dermatol* 1973; 60: 443–456.
132 White JG, Witkop CJ Jr, Effects of normal and aspirin platelets on defective secondary aggregation in the Hermansky-Pudlak syndrome: A test for storage pool deficient platelets. *Am J Pathol* 1972; 68: 57–66.
133 Gerrard JM, White JG, Rao GHR, Krivit W, Witkop CJ Jr. Labile aggregation stimulating substance (LASS): The factor from storage pool deficient platelets connecting defective aggregation and release of aspirin treated normal platelets. *Br J Haematol* 1975; 29: 657–665.
134 Breton-Gorius J, Guichard J. Ultrastructural localization of peroxidase activity in human platelets and megakaryocytes. *Am J Pathol* 1972; 66: 277–286.
135 Gerrard JM, White JG, Rao GHR, Townsend D. Localization of platelet prostaglandin production in the platelet dense tubular system. *Am J Pathol* 1976; 83: 283–298.
136 Carey F, Menashi S, Crawford N. Localization of cyclooxygenase and thromboxane synthetase in human platelet intracellular membranes. *Biochem J* 1982; 204: 847–851.
137 Gerrard JM, White JG, Peterson DA. The platelet dense tubular system: Its relationship to prostaglandin synthesis and calcium flux. *Thromb Haemost* 1978; 40: 224–231.

Cambridge Books Online © Cambridge University Press, 2009

4

Platelet heterogeneity: physiology and pathological consequences

Anthony Mathur and John F. Martin

Centre for Cardiovascular Biology and Medicine, University College London, UK

Introduction

Individual platelets vary in terms of volume, density and reactivity. Ever since the seminal paper by Karpatkin[1], the biological significance of this platelet heterogeneity has been strongly contested.

Platelet volume, usually measured as mean platelet volume (MPV), has been the most frequently studied platelet physical variable. During steady-state hematopoeisis the platelet volume distribution approaches log normality with platelets ranging in size from $<2\ \mu m^3$ to $>15\ \mu m^3$ in volume. This variation in size is considerably greater than that observed for other circulating blood elements in any mammal. This heterogeneity forms the basis of the controversy with respect to the physiological relevance of platelet volume and reactivity[2].

Separation of platelets into size dependent subpopulations[3] has shown that the granular contents of these cells is directly proportional to their volume. Importantly, the ability of platelets to affect each other and their environment as measured by aggregation and total amount of granular contents released, is proportional to their size and confirmed by the finding that platelet volume correlates with absolute platelet function[4,5]. The physiological explanation for this lies in the observation that high-density platelets contain significantly higher concentrations of alpha granule proteins (β thromboglobulin, von Willebrand factor), dense granule markers (5-HT, calcium), mitochondrial marker enzymes (monamine oxidase, cytochrome oxidase, glutamate dehydrogenase and NADP-dependent isocitrate dehydrogenase), glycogen[6,7] and express more of the GPIIb/IIIa receptor[8].

Furthermore although only limited work has been performed on functional properties of platelet density subpopulations, Karpatkin's original report[1] showed that high-density platelets aggregated more quickly and released greater quantities of ATP, ADP and platelet factor 4 than low-density platelets in response to stimulation by ADP, thrombin and adrenaline (although the use of non-linear gradients in this work to determine platelet density-dependent subpopulations has been questioned by Martin & Trowbridge[9]).

However more recent work has shown that, although high-density platelets may produce more thromboxane β_2 in response to 0.5 mmol arachidonate than low-density platelets, response to thrombin stimulation remains the same[10]. Nubile et al.[11] reported a greater extent of aggregation in response to ADP in low-density platelets compared to high-density platelets, while the response to collagen and adrenaline was slightly greater in high-density platelets. Other groups have also reported no differences in 5-HT transport between platelet populations and, importantly, that all platelet populations lose similar percentages of their dense bodies in respect to thrombin stimulation[12].

This would suggest that any increase in function as measured by biochemical markers is solely due to the increase in platelet contents that occurs in proportion to their density and size and not due to an overproduction of these factors within high-density platelet populations.

Causes of heterogeneity

Two separate hypotheses which are mutually exclusive have been proposed to explain the origin of platelet heterogeneity. The first maintains that changes in platelet properties are the result of the ageing of platelets in the circulation. The second holds that platelets are created with properties that remain unchanged as the platelet ages. Both these possibilities shall be considered individually below.

Platelet ageing as a cause of heterogeneity

Considerable work, by several groups, has demonstrated that platelet density increases[13–15] decreases[1,16–22] or remains stable[14,23,24] as platelets age. Various models have been used to study the phenomenon of platelet ageing. Mezzano et al.[13] measured the survival of ^{51}Cr labelled unfractionated human platelets and radio labelled density cohorts. They concluded that newly formed platelets become denser with platelet ageing. They also found that platelet survival of fractions enhanced with low-density platelets were significantly longer than that of fractions enhanced with high-density platelets, data confirmed by Martin and Penington[14] using *Macaca fasicularis* monkeys. However, in another study using radio-labelled density dependent platelet cohorts in splenectomized dogs, they found that, in contrast to human studies, platelet density in dogs decreases with ageing. This confirmed work performed by Corash's group[17] using *Macaca mulata* monkeys in which high-density and low-density platelet cohorts were isolated, labelled with radiochromium (^{51}Cr), reinjected into donor animals and the survival and density distribution of the labelled cohorts followed with serial density gradient analyses. Corash and Shafer[18] also used another experimental model of injecting radio-labelled amino acids into rabbits followed by serial fractionation of platelets into density dependent platelet cohorts to again confirm that young platelets tend to be high-density and that with age, density decreased. Evidence to support the suggestion that platelet density does not change with ageing comes from observations of patients in whom thrombopoiesis could be switched on by plasma infusion[25]. Relatively young platelets were obtained 4 days after infusion and older platelets 18–21 days after infusion. Although older platelets were associated with longer bleeding times, decreased platelet adhesiveness and deficient platelet factor 3 availability, no structural differences between young and old platelets were observed by electron microscopy.

Thus, although platelets are heterogeneous with respect to size and density, evidence exists that links density changes with both young and old platelets suggesting that density is independent of ageing. Several groups[5,12] have used discontinuous gradients to separate platelets by density, but when Martin's group used this technique[9] they found that platelet populations taken from each 'density' interface comprised platelets with a variety of densities. The authors therefore questioned the validity of discontinuous gradients to measure absolute platelet densities. These observations vary depending upon the type of model used and method used for separating and determining platelet density. The fact that there are many different interpretations of these observations is in keeping that platelet heterogeneity is not related to platelet age and hence the second hypothesis.

Thrombopoiesis as the origin of platelet heterogeneity

Observations made during steady-state thrombopoiesis have shown that MPV and platelet counts are inversely related, suggesting that platelet production is regulated to maintain a constant circulating functional platelet mass[26]. This hypothesis has been tested by observing platelet count and MPV in Rhesus monkeys during continuous infusion of granulocyte – macrophage colony stimulating factor (GM–CSF). Infusion of GM–CSF leads to a significant increase in circulating platelet count and concomitant decline in MPV[27]. Other studies[28–32] have shown that stimulated thrombopoiesis, which occurs during periods of increased haemostatic demand can induce increases in firstly MPV and secondly number of platelets produced. Linking these observations to a mechanism that controls platelet size at the time of their production are the experiments of Corash et al.[29] using a murine model to examine the relationship between bone marrow megakaryocyte (MK) ploidy and platelet volume. In response to acute severe thrombocytopenia (reduction in platelet level of $<0.05\times10^6/\mu l$), platelet volume was noted to increase 40 hours before a detectable shift in bone marrow MK ploidy[29]. Less severe thrombocytopenia (reduction of the platelet level to 0.015 to $0.25\times10^6/ml$) resulted in a delay of the shift in ploidy distribution from 48 to 72 hours after the onset of thrombocytopenia. Moderate thrombocytopenia ($0.30–0.4\times10^6/\mu l$) did not result in a ploidy shift, but did produce a significant and prolonged increment in MPV. In response to moderate thrombocytopenia, MPV was significantly increased as early as 12 hours after administration of platelet antiserum and remained increased during the period of thrombocytosis following recovery[29]. These results lead Corash et al. to suggest that changes in MPV occur before any changes in MK ploidy but that these changes may arise secondarily to changes in the actual platelet release process. However, Martin and Penington[14] have shown that MK ploidy was found to increase in the presence of platelet destruction and that large platelets were released into the circulation. These platelets were found to have increased thromboxane A_2 production per unit volume of platelet cytoplasm suggesting a preceding change in MK cytoplasmic reactivity occuring within 24 hours following platelet destruction. These platelets

Cambridge Books Online © Cambridge University Press, 2009

reduced bleeding time more than controls per unit volume cytoplasm[33]. Changes in mean bone marrow MK ploidy are slow and associated with an increase in MK cytoplasmic volume[34]. Such a change is associated with a sustained increase in platelet volume when platelets are continuously destroyed to reveal the size of the newly produced platelets[35]. However, an increase in ploidy appears to be associated consistently with an increase in platelet count[23,34,36–38]. Kristensen et al.[39] have shown that in man, under normal conditions, there is a significant negative correlation between MK ploidy and bleeding time (which is determined by platelet behaviour). However, platelet volume was not related to MK ploidy in these normal individuals during steady-state platelet production. If changes in MK ploidy are induced without a change in the rate of destruction, e.g. by injection of vincristine, thrombocytosis occurs without a change of platelet volume[40]. These data therefore argue that the origins of platelet heterogeneity lie in the highly regulated and complicated process of thrombopoiesis itself as opposed to changes occurring in the circulation as platelets age.

Thrombopoietin (TPO) has been described as the most potent hormonal regulator of platelet production in man[41]. Its discovery, however, has occurred several years after most of the work on platelet heterogeneity was published. It is now thought that TPO is constitutively produced by the liver and kidneys[42] and that the combined MK/platelet mass is kept at a constant level by self-regulatory mechanism involving the TPO receptor c-mpl found on platelets and MKs. It is probable that TPO controls platelet number by causing changes in MK ploidy, although there is still no evidence concerning the controlling mechanism of platelet volume.

Theories of platelet production

Not only is the mechanism of platelet production disputed, but also the site.

MK fragmentation in the bone marrow

Wright in 1906 through observations of fixed sections of rat bone marrow, using a light microscope, first described loss of MK cytoplasm by detachment of buds of platelet-like fragments[43]. The presence of MKs with pseudopodia, in the bone marrow, has been confirmed by electron microscopy[44–48]. This subject has recently been revisited by Zucker-Franklin again to demonstrate in a static system filamentous extensions of MK cytoplasm[49]. Filamentous extensions of MK cytoplasm have also been observed in culture[50–53]. These have been thought to correspond to the cytoplasmic protrusions seen by Wright[43,54]. Taken together these observations would suggest that platelets are actively produced in the bone marrow. However, MKs have never been observed producing platelets in the marrow and these conclusions may result from artefacts related to in vitro conditions or preparation methods. Given that, in general, the majority of hematopoiesis occurs within the bone marrow, the concept that thrombopoiesis occurs at this site still remains a logical possibility, but lacks definitive evidence.

Platelet production from the demarcation membrane system (DMS)

MKs are approximately 10–500 times as large as a human red blood cell. Approximately four-fifths of this volume is cytoplasm, which contains various organelles. Observations of the contents of MK cytoplasm by Yamada[55] led to the proposal of an extensively invaginated demarcation membrane system (DMS) that delineated future platelets in the MK cytoplasm. The DMS appears as a tubular arrangement[56] but does not surround cytoplasm and therefore, by itself, cannot delineate platelets. Thus the suggestion by Shaklai and Tavassoli of a mechanism of tubular fusion followed by membrane fission was put forward to explain how a tubular system can be transformed into the flat membranes that are necessary for the enclosure of the platelet cytoplasm. Rather than the DMS, 'platelet territories' were therefore proposed as the site of platelet production in the MK. Further morphological data suggested that DMS was a misnomer and that the tubular invaginations act as a membrane reserve for the attenuated processes. However, this suggestion has been subsequently thrown into confusion following freeze–fracture studies of Shaklai and Tavassoli that showed the invaginated system to consist of a complex of interacting channels involving fusion and branching so that there is communication between many of the invaginations. White[57] has suggested that this complex of intracytoplasmic tunnels would be torn apart in simple evagination. Thus several inconsistencies appear in the platelet territory explanation of platelet production. Despite these problems, the process of growth and demarcation of platelet territories has been proposed as a mechanism for platelet volume heterogeneity which would explain the platelet log volume distribution[58]. This model does not address the problem of platelet release from the MK nor does it identify the mechanism of biological control over growth and demarcation which is supposed to determine platelet size.

Cambridge Books Online © Cambridge University Press, 2009

Platelet production in the peripheral circulation

Electron microscopic evidence that MKs in their entirety migrate through the vessel wall[59] suggests that platelet production may occur at a site away from the bone marrow. It has also been demonstrated that megakaryocytic cytoplasm is capable of amoeboid movement[60], leaving the naked nucleus behind in the marrow. These observations suggest that both MKs and megakaryocytic cytoplasmic processes are capable of transendothelial migration into the bone marrow sinusoids and hence into the peripheral circulation. Direct evidence is also produced by the work of Kinosita and Ohno[61] in which windows were made in the femur of rabbits and the marrow tissue, once it had regenerated, examined by phase contrast microscopy. In these experiments, whole MKs were observed in the bone marrow sinusoids, but cytoplasmic processes were never observed projecting into the sinusoidal lumen. It was also noted that platelets were produced by cytoplasmic disintegration in the extrasinusoidal and sinusoidal spaces although the possibility that this was due to MK spreading on the glass of the windows cannot be excluded.

Circulating MKs (of the order of 25 MKs per ml of blood) have been found in the adult human vena cava[62]. Differential concentrations of cytoplasm bearing MKs have been demonstrated between arterial and venous blood (increased concentrations on the venous side) suggesting that either some filtering of MKs occurs between central venous blood and arterial blood[62] or that the MKs in venous blood fragment to form platelets[63]. Similar findings have been reported in other mammals including rat, dog and rabbits[64–66] suggesting potentially similar mechanisms of platelet production. This is logical since all mammals have a $\log_{10}$ normal distribution of platelet volume and polyploidy[67,68]. Due to the difficulties of studying MKs as they circulate, it is difficult to obtain evidence that shows platelet production in circulating blood. However, the pulmonary capillaries act as filters for large cells, and it has been suggested that they trap circulating MKs and form platelets at this site. The evidence for this shall be considered below.

Platelet production from circulating MKs in the lungs

An alternative theory regarding the site of platelet production was developed from observations made as long ago as 1893 by Aschoff[69] and subsequently by Wright in 1906[43], of the presence of MKs in the pulmonary vessels. Since these MKs were found mainly to consist of naked cytoplasmic material, the general opinion was that the MKs were migrants from the bone marrow (or spleen in some circumstances) which had lost their cytoplasm in the process of platelet formation before reaching the lungs, or shortly after being trapped in the pulmonary capillaries.

However, these observations were questioned following the observation of cytoplasmic processes extending from pulmonary MKs and pushing through pulmonary capillaries[70]. This data therefore seemed incompatible with the notion of pulmonary MKs representing degenerating cells that had been cast off from the bone marrow and it was postulated that MKs are formed or develop in the lungs themselves from myeoblastic cells.

Autopsy studies revealed the presence of pulmonary MKs in every one of 50 cases[71] as well as in other organs such as spleen, liver, kidney and heart. This led to the suggestion that these MKs originate in the bone marrow and their presence in other organs is therefore dependent upon the number of MKs entering the systemic circulation or the bone marrow and subsequently passing through the pulmonary bed to reach the other organs.

Experiments in dogs whereby blood to the left lung was shunted, bypassing systemic circulation, revealed that the lung receiving systemic blood (that had passed through the bone marrow) had almost 100 times higher concentration of MKs than the shunted lungs[72]. This therefore refuted the belief that MKs formed within lung tissue but still left the possibility of platelet production within the pulmonary capillary bed.

Other authors have also reported the presence of mature MKs in pulmonary arteries and the presence of naked nuclei in the pulmonary capillary[48,73]. They have also demonstrated a relationship between pulmonary MKs and microthrombosis, suggesting that conditions that involve generalized thrombus formation (e.g. disseminated intravascular coagulation) were also associated with increased concentrations of pulmonary MKs[73].

Thus considerable evidence suggests that MKs are found in pulmonary capillaries but what becomes of them? One possibility is that they change shape and pass through the pulmonary capillaries into the systemic circulation. Observational evidence suggests that this is not the case due to the finding of naked nuclei in the pulmonary bed[62] and the decreased amount of MKs with cytoplasm found in the systemic arterial circulation compared to the venous circulation[62,74]. The loss of MK cytoplasm within the pulmonary bed has led to the development of mathematical models to calculate MK cytoplasmic volume distribution in an attempt to demonstrate that sufficient MK cytoplasmic material reaches the lungs to account for circulating platelet counts[75,76]. Furthermore, the model

Cambridge Books Online © Cambridge University Press, 2009

proposes that platelet production through MK fragmentation involves two stochastic processes that would produce heterogeneity in the circulating platelet population. The log-normal platelet volume distribution would be explained by the fact that multiple fragmentation steps led to such a distribution in all physical fragmentation processes.

Pathophysiology

So far, the origins and mechanisms leading to platelet heterogeneity have been considered. An important question is what role if any does platelet heterogeneity play in human disease?

Since platelets are involved in arterial thrombus formation and drugs that inhibit platelet behaviour are beneficial in preventing arterial occlusion it is of interest that alterations in platelet size and density have been described in arterial disease[77–80]. Examination of platelet physiology in these conditions has produced interesting data that has led to some understanding of the role of platelet heterogeneity in the human. Platelet density has been extensively studied in the human suffering from myocardial infarction. Since myocardial infarction nearly always occurs in the presence of atherosclerosis, this disease provides a convenient means of studying the alterations in platelet size and density previously described. Martin et al.[79] discovered that the density of platelets from patients with myocardial infarction was significantly higher than from control subjects. This study also demonstrated a concomitant increase in platelet volume that has been confirmed by other authors[77,78]. Since large platelets are more reactive than small platelets[1,3,33,81], it is reasonable to suggest that if large platelets are present in the circulation before myocardial infarction, their presence may be causally linked to the occurrence of thrombosis in the coronary artery. In acute myocardial infarction, not only is the average MPV increased but also the whole of the platelet volume distribution is shifted to the right[82] (Fig. 4.1).

If the platelet volume distribution curve has its origin in thrombopoiesis, then this implies a change in the MK in myocardial infarction. Models describing the control of thrombopoiesis (outlined below) suggest that a low platelet count and large platelet volume (as found in acute myocardial infarction) may follow acute platelet destruction[9] or chronic slow platelet destruction[84]. In both these cases there is a change to larger, higher ploidy MKs, although the large platelets following acute destruction occur before changes in the MK ploidy[29,34].

Bone marrow biopsy of patients following acute myocardial infarction have confirmed that MKs are bigger than those from controls[80]. Autopsy studies also demonstrated that MKs in patients who suffered a sudden cardiac death were larger than from patients who died due to trauma, and had normal coronary arteries[80]. Since large MKs are associated with any increase in the rate of platelet production, the large platelets in acute myocardial infarction probably occur secondary to the stimulus from an increase in platelet destruction.

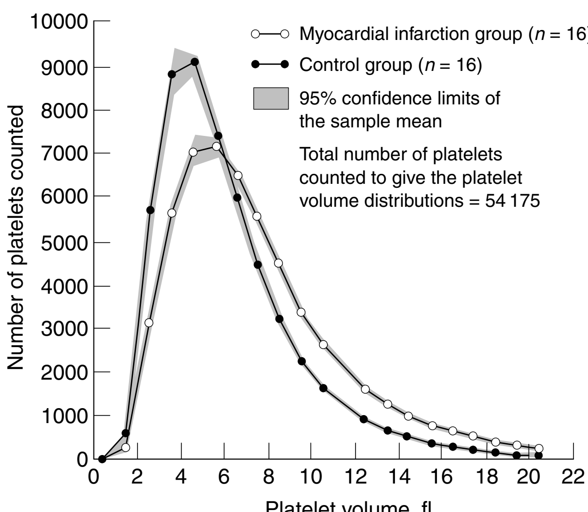


Fig. 4.1. Average platelet volume distribution of patients with acute myocardial infarction (open circles) and controls (closed circles). Note that the whole platelet volume distribution curve is shifted to higher values in myocardial infarction compared with controls. Since the log normal curve of platelet distribution has its origin in thrombopoiesis this shift probably implies a change in thrombopoiesis in myocardial infarction[82]. Although these platelets were taken from patients after myocardial infarction there is evidence in second myocardial infarction that these changes have preceded this second event[83].

The significance of an increase in platelet size in acute myocardial infarction has been studied in relation to the bleeding time. Milner and Martin[85] demonstrated that mean bleeding time in acute myocardial infarction is shortened compared to controls. It is also of interest that bleeding time was found to be inversely related to MK size and ploidy in normal subjects[39]. Thus the finding of a shortened bleeding time, large platelets and large MKs in acute myocardial infarction provides consistent independent evidence that these factors may be causally related to the acute event. Furthermore, in a study of 2000 men followed over 2 years, increases in platelet size 6 months after their first myocardial infarction was the strongest predictor of a second myocardial infarction or death[83]. Since these changes occurred before myocardial infarction, it is pos-

Cambridge Books Online © Cambridge University Press, 2009

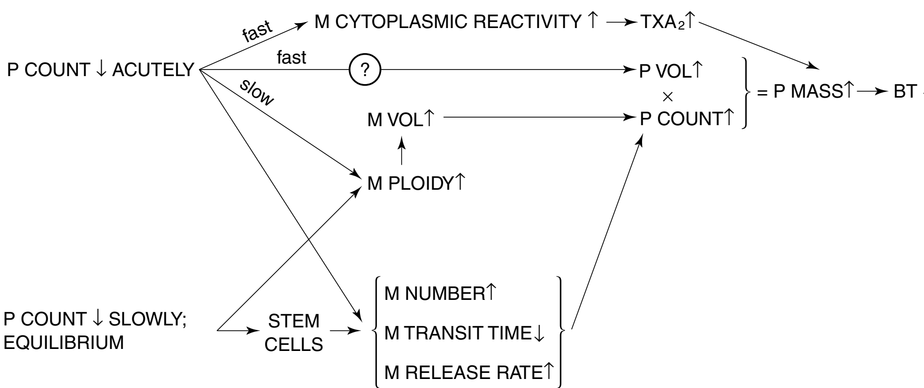


Fig. 4.2. Proposed system of relationships between change in platelet destruction rate, alteration in MK variables, change in platelet mass and bleeding time. P = platelet, M = megakaryocyte, TXA_2 = thromboxane A_2, BT = bleeding time, vol = volume, ↑ = increased, ↓ = decreased, → = an association[35].

sible that changes in platelet volume precede and may be causally related to the first myocardial infarction (in that subpopulation of the syndrome where platelet reactivity is a cause of coronary artery occlusion – reviewed in Van der Loo and Martin[86]).

Since platelet destruction is associated with increases in MK ploidy[23,29,31,38,87] and platelet hypertransfusion with decreases in ploidy[36,38], it could be concluded that ploidy change is the mechanism by which circulating platelet counts may be adjusted, probably to adjust to hemostatic needs. Thus presupposing that a change in platelet destruction rate is the event that signals changing hemostatic need and that in response, change in bleeding time is the ultimate physiological manifestation of the changes in cellular hemostasis, a mechanism controlling platelet production is outlined above (Fig. 4.2).

As already mentioned, in most acute myocardial infarction, platelet counts are reduced, producing a stimulus for the mechanism outlined above. Recently, our work has also explored the relationship of MPV between acute myocardial infarction and unstable angina[88] following on from an earlier study that demonstrated an increase in MPV in patients with unstable angina compared with controls[89]. We found that, in unstable angina, MPV is increased relative to that in acute myocardial infarction, and that platelet count is decreased. Although both conditions demonstrated an increase in expression of surface markers of platelet activation compared to controls, these variables were increased more in myocardial infarction than in unstable angina. Whilst these observations may be explained by postulating that platelet changes in unstable angina are due to a more chronic process of platelet destruction compared to acute myocardial infarction, it does not explain why the large platelets of unstable angina are not associated with an increase in activation or a decrease in bleeding time[90]. Little data exist that describe changes in bone marrow MKs in unstable angina, and without this part of the puzzle it is difficult to fully explain these findings.

Various models have been used in an attempt to study changes in platelet physiology in response to platelet destruction. Injection of antiplatelet antibodies into rats and mice has shown an increase in platelet volume several hours later[29,91]. A useful model of platelet destruction in man results from the study of changes in platelet parameters of patients undergoing cardio-pulmonary bypass at heart valve replacement. This demonstrates a rise in MPV with a fall in platelet count (Figure 4.3) induced by the operation[92]. Other operations[93] have also demonstrated this relationship as well as an increase in TPO levels in response to the decrease in platelet count, providing further evidence for the role of TPO in the control of platelet volume.

Conclusions

The significance of platelet heterogeneity still remains controversial in spite of the wealth of evidence that has been collected. This lack of full understanding of normal physiology is a major drawback to understanding several disease processes such as myocardial infarction. Although observational data has been collected from humans, without the ability to manipulate the system, this cannot be taken further. What we understand so far is that platelets are heterogeneous with respect to size and this may

Cambridge Books Online © Cambridge University Press, 2009

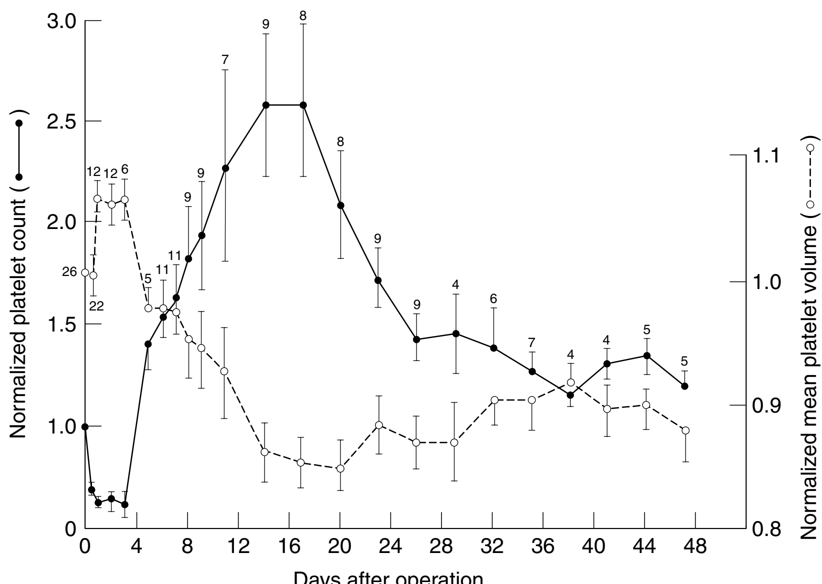


Fig. 4.3. The changes in platelet count and mean platelet volume following cardiopulmonary bypass surgery[92].

well reflect changes in platelet function. These changes probably occur at the time of platelet production, although the exact site at which platelets are produced remains controversial. Large platelets are denser and are functionally more reactive than small platelets. Changes in platelet size and density are not necessarily preceded by changes in MK size and ploidy, although these do increase with an increase in the size of circulating platelets.

Patients with acute myocardial infarction and unstable angina are found to have platelets with increased MPV. Increased MPV is associated with increased expression of surface markers of platelet activation in both of these conditions. Increased MK size and ploidy have been found in acute myocardial infarction suggesting that changes in platelet function may causally be related to acute coronary syndromes. The relationship between changes in platelet size and count post surgery with TPO levels would suggest that changes in TPO levels may control platelet behaviour and that these changes occur in an attempt to maintain haemostatic equilibrium.

This suggests that if changes in platelet physiology are causal to acute coronary syndromes, these may occur as a result of the pathological transformation of a physiological protective mechanism. The trigger for this transformation has yet to be determined.

References

1. Karpatkin S. Heterogeneity of human platelets. I. Metabolic and kinetic evidence suggestive of young and old platelets. *J Clin Invest* 1969a; 48: 1073–1082.
2. Paulus JM. Platelet size in man. *Blood* 1975; 46: 321–336.
3. Thompson CB, Eaton KA, Princiotta SM, Rushin CA, Valeri CR. Size dependent platelet subpopulations: relationship of platelet volume to ultrastructure, enzymatic activity, and function. *Br J Haematol* 1982; 50: 509–519.
4. Mannucci PM, Sharp AA. Platelet volume and shape in relation to aggregation and adhesion. *Br J Haematol* 1967; 13: 604–617.
5. Karpatkin S. Heterogeneity of human platelets. VI. Correlation of platelet function with platelet volume. *Blood* 1978; 51: 307–316.
6. Chamberlain KG, Penington DG. Monoamine oxidase and other mitochondrial enzymes in density subpopulations of human platelets. *Thromb Haemost* 1988; 59: 29–33.
7. Chamberlain KG, Seth P, Jones MK, Penington DG. Subcellular composition of platelet density subpopulations prepared using continuous Percoll gradients. *Br J Haematol* 1989; 72: 199–207.
8. Giles H, Smith RE, Martin JF. Platelet glycoprotein IIb–IIIa and size are increased in acute myocardial infarction. *Eur J Clin Invest* 1994; 24: 69–72.
9. Martin JF, Trowbridge EA. Theoretical requirements for the density separation of platelets with comparison of continuous and discontinuous gradients. *Thromb Res* 1982; 27: 513–522.
10. Martin JF, Shaw T, Jakubowski JA, Penington DG, Martin TJ. Production of thromboxane B2 by platelets is related to their density. *Thromb Haemost* 1981a; 46: 198.

Cambridge Books Online © Cambridge University Press, 2009

11 Nubile G, Di Gregorio M, Ridolfi D, D'Alonzo L, Izzi L. [Platelet aggregation induced by adenosine diphosphate, collagen and adrenaline in human platelet subpopulations]. *Quad Sclavo Diagn* 1987; 23: 331–340.

12 Corash L, Costa JL, Shafer B, Donlon JA, Murphy D. Heterogeneity of human whole blood platelet subpopulations. III. Density-dependent differences in subcellular constituents. *Blood* 1984; 64: 185–193.

13 Mezzano D, Hwang K, Catalano P, Aster RH. Evidence that platelet buoyant density, but not size, correlates with platelet age in man. *Am J Hematol* 1981; 11: 61–76.

14 Martin JF, Penington DG. The relationship between the age and density of circulating 51–Cr labelled platelets in the subhuman primate. *Thromb Res* 1983; 30: 157–164.

15 Savage B, McFadden PR, Hanson SR, Harker LA. The relation of platelet density to platelet age: survival of low- and high-density 111indium-labeled platelets in baboons. *Blood* 1984; 68: 386–393.

16 Amorosi E, Garg SK, Karpatkin S. Heterogeneity of human platelets. IV. Identification of a young platelet population with Se75-selenomethionine. *Br J Haematol* 1971; 21: 227–232.

17 Corash L, Shafer B, Perlow M. Heterogeneity of human whole blood platelet subpopulations. II. Use of a subhuman primate model to analyze the relationship between density and platelet age. *Blood* 1978; 52: 726–734.

18 Corash L, Shafer B. Use of asplenic rabbits to demonstrate that platelet age and density are related. *Blood* 1982; 60: 166–171.

19 Mezzano D, Aranda E, Foradori A, Rodriguez S, Lira P. Kinetics of platelet density subpopulations in splenectomized mongrel dogs. *Am J Hematol* 1984; 17: 373–382.

20 Packham MA, Guccione MA, O'Brien KM. Duration of the effect of aspirin on the synthesis of thromboxane by density subpopulations of rabbit platelets stimulated with thrombin. *Blood* 1985; 66: 287–290.

21 Rand ML, Greenberg JP, Packham MA, Mustard JF. Density subpopulations of rabbit platelets: size, protein, and sialic acid content, and specific radioactivity changes following labeling with 35S-sulfate in vivo. *Blood* 1981; 57: 741–746.

22 Rand ML, Packham MA, Mustard JF. Survival of density subpopulations of rabbit platelets: use of 51Cr-or 111In-labeled platelets to measure survival of least dense and most dense platelets concurrently. *Blood* 1983; 61: 362–367.

23 Martin JF, Shaw T, Heggie J, Penington DG. Measurement of the density of human platelets and its relationship to volume. *Br J Haematol* 1983c; 54: 337–352.

24 Penington DG, Lee NLY, Roxburgh AE, McGready JR. Platelet density and size: the interpretation of heterogeneity. *Br J Haematol* 1976; 34: 365–376.

25 Johnson CA, Abildgaard CF, Schulman I. Functional studies of young versus old platelets in a patient with chronic thrombocytopenia. *Blood* 1971; 37: 163–171.

26 Thompson CB, Jakubowski JA. The pathophysiology and clinical relevance of platelet heterogeneity. *Blood* 1988; 72: 1–8.

27 Monroy RL, Skelly RR, MacVittie TJ et al. The effect of recombinant GM-CSF on the recovery of monkeys transplanted with autologous bone marrow. *Blood* 1987; 70: 1696–1699.

28 Bessman JD. The relation of megakaryocyte ploidy to platelet volume. *Am J Hematol* 1984; 16: 161–170.

29 Corash L, Chen HY, Levin J, Baker G, Lu H, Mok Y. Regulation of thrombopoiesis: effects of the degree of thrombocytopenia on megakaryocyte ploidy and platelet volume. *Blood* 1987; 70: 177–185.

30 Evatt BL, Levin J. Measurement of thrombopoiesis in rabbits using 75selenomethionine. *J Clin Invest* 1969; 48: 1615–1626.

31 Martin JF, Trowbridge EA, Salmon GL, Slater DN. The relationship between platelet and megakaryocyte volumes. *Thromb Res* 1982; 28: 447–459.

32 Tong M, Seth P, Penington DG. Proplatelets and stress platelets. *Blood* 1987; 69: 522–528.

33 Martin JF, Trowbridge EA, Salmon G, Plumb J. The biological significance of platelet volume: its relationship to bleeding time, platelet thromboxane B2 production and megakaryocyte nuclear DNA concentration. *Thromb Res* 1983a; 32: 443–460.

34 Warren CW. The thrombopoietic control system: homeostasis, simulation and pertubation. 1987; PhD thesis, University of Sheffield.

35 Martin J. The relationship between megakaryocyte ploidy and platelet volume. *Blood Cells* 1989; 15: 108–121.

36 Harker LA. Kinetics of thrombopoiesis. *J Clin Invest* 1968; 47: 458–465.

37 Odell TT, McDonald M, Detwiler TC. Stimulation of platelet production by serum of platelet-depleted rats. *Proc Soc Exp Biol Med* 1961; 108: 428–431.

38 Penington DG, Olsen TE. Megakaryocytes in states of altered platelet production: cell numbers, size and DNA content. *Br J Haematol* 1970; 18: 447–463.

39 Kristensen SD, Bath PM, Martin JF. The bleeding time is inversely related to megakaryocyte nuclear DNA content and size in man. *Thromb Haemost* 1988; 59: 357–359.

40 Martin JF, Francis P, Lee L, Shaw T, Macpherson J, Penington DG. Differences in the volume of stress platelets and vincristine stimulated platelets. *Thromb Haemost* 1981b; 46: 410

41 Kaushansky K. The mpl ligand: molecular and cellular biology of the critical regulator of megakaryocyte development. *Stem Cells* 1994; 12 Suppl 1: 91–96.

42 Cohen-Solal K, Villeval JL, Titeux M, Lok S, Vainchenker W, Wendling F. Constitutive expression of Mpl ligand transcripts during thrombocytopenia or thrombocytosis. *Blood* 1996; 88: 2578–2584.

43 Wright JH. The origin and nature of the blood plates. *Boston Med Surg J* 1906; 154: 643–645.

44 Becker RP, De Bruyn PP. The transmural passage of blood cells into myeloid sinusoids and the entry of platelets into the sinusoidal circulation; a scanning electron microscopic investigation. *Am J Anat* 1976; 145: 183–205.

45 Behnke O. An electron microscopic study of the rat megakaryocyte. II. Some aspects of platelet release and microtubules. *J Ultrastruct Res* 1969; 26: 111–129.

46 Lichtman MA, Chamberlain JK, Simon W, Santillo PA.

Cambridge Books Online © Cambridge University Press, 2009

Parasinusoidal location of megakaryocytes in marrow: a determinant of platelet release. *Am J Hematol* 1978; 4: 303–312.
47 Muto M. A scanning and transmission electron microscopic study on rat bone marrow sinuses and transmural migration of blood cells. *Arch Histol Jpn* 1976; 39: 51–66.
48 Slater DN, Trowbridge EA, Martin JF. The megakaryocyte in thrombocytopenia: a microscopic study which supports the theory that platelets are produced in the pulmonary circulation. *Thromb Res* 1983a; 31: 163–176.
49 Zucker-Franklin D, Philipp CS. Platelet production in the pulmonary capillary bed: new ultrastructural evidence for an old concept. *Am J Pathol* 2000; 157: 69–74.
50 Haller CJ, Radley JM. Time-lapse cinemicrography and scanning electron microscopy of platelet formation by megakaryocytes. *Blood Cells* 1983; 9: 407–418.
51 Radley JM, Scurfield G. The mechanism of platelet release. *Blood* 1980; 56: 996–999.
52 Radley JM, Hartshorn MA. Megakaryocyte fragments and the microtubule coil. *Blood Cells* 1987; 12: 603–614.
53 Thiery JP, Bessis M. La genese des plaquettes sanguines a partir des megakaryocytes observee sur la cellule vivante. *R Acad Sci* 1956; 242: 290–292.
54 Wright JH. The histogenesis of the blood platelets. *J Morphol* 1910; 21:263–278.
55 Yamada E. The fine structure of the megakaryocyte in the mouse spleen. *Acta Anat* 1957; 29: 267–290.
56 Shaklai M, Tavassoli M. Demarcation membrane system in rat megakaryocyte and the mechanism of platelet formation: a membrane reorganization process. *J Ultrastruct Res* 1978; 62: 270–285.
57 White JG. Time-lapse cinemicrography and scanning electron microscopy of platelet formation by megakaryocytes: a commentary. *Blood Cells* 1983; 9: 419–426.
58 Paulus JM, Bury J, Grosdent JC. Control of platelet territory development in megakaryocytes. *Blood Cells* 1979; 5: 59–88.
59 Tavassoli M, Aoki M. Migration of entire megakaryocytes through the marrow–blood barrier. *Br J Haematol* 1981; 48: 25–29.
60 Slater DN, Martin JF, Trowbridge EA. The lung: a platelet factory and megakaryocyte graveyard in health and disease. *J Pathol* 1983b; 140: 144
61 Kinosita R, Ohno S. Biodynamics of thrombopoiesis. In Johnson SA, Monto RW, Rebuck JW, Horn RC. *Blood Platelets.* London: Churchill.
62 Pedersen NT. Occurrence of megakaryocytes in various vessels and their retention in the pulmonary capillaries in man. *Scand J Haematol* 1978; 21: 369–375.
63 Trowbridge EA, Martin JF, Slater DN. Evidence for a theory of physical fragmentation of megakaryocytes, implying that all platelets are produced in the pulmonary circulation. *Thromb Res* 1982; 28: 461–475.
64 Aabo K, Hansen KB. New aspects of the morphology of circulating megakaryocytes in rabbits. *Acta Pathol Microbiol Scand [A]* 1979; 87A: 173–177.
65 Pedersen NT. Circulating megakaryocytes in blood from the inferior vena cava in adult rats. *Scand J Haematol.* 1971; 8: 223–230.
66 Warheit DB, Barnhart MI. Circulating megakaryocytes and the microvasculature of the lung. *Scan Electron Microsc* 1980; 3: 255–262.
67 von Behrens WE. Megakaryocyte polyploidy as a grouped geometric distribution obeying the log-normal population law. *Int J Cell Cloning* 1990; 8: 307–315.
68 Martin JF. Punctuated equilibrium in evolution and the platelet. *Eur J Clin Invest* 1994; 24: 291
69 Aschoff L. Uber capillare embolie von mesenkernzellen. *Virch Arch Path Anat Physiol Klin Med* 1893; 134: 11–24.
70 Howell WH, Donahue DD. The production of blood platelets in the lungs. *J Exp Med* 1937; 65: 177–203.
71 Brill R, Halpern MM. The frequency of megakaryocytes in autopsy sections. *Blood* 1948; 3: 286–291.
72 Kaufman RM, Airo R, Pollack S, Crosby WH, Doberneck R. Origin of pulmonary megakaryocytes. *Blood* 1965; 25: 767–775.
73 Hansen KB, Aabo K. Megakaryocytes in pulmonary blood vessels. *Acta Pathol Microbiol Scand [A]* 1978; 86: 293–295.
74 Shoff PK, Kirwin KS, Levine RF. Megakaryocytes in circulating blood. *Circulation.* 1987; 76: 1339(Abstract).
75 Trowbridge EA, Martin JF, Slater DN et al. The origin of platelet count and volume. *Clin Phys Physiol Meas* 1984b; 5: 145–170.
76 Trowbridge EA, Harley PJ. A stochastic model of pulmonary platelet production. *IMA J Math Appl Med Biol* 1988; 5: 45–63.
77 Cameron HA, Phillips R, Ibbotson RM, Carson PH. Platelet size in myocardial infarction. *Br Med J (Clin Res Ed)* 1983; 287: 449–451.
78 Kishk YT, Trowbridge EA, Martin JF. Platelet volume subpopulations in acute myocardial infarction: an investigation of their homogeneity for smoking, infarct size and site. *Clin Sci (Colch)* 1985; 68: 419–425.
79 Martin JF, Plumb J, Kilbey RS, Kishk YT. Changes in volume and density of platelets in myocardial infarction. *Br Med J (Clin Res Ed)* 1983b; 287: 456–459.
80 Trowbridge EA, Slater DN, Kishk YT, Woodcock BW, Martin JF. Platelet production in myocardial infarction and sudden cardiac death. *Thromb Haemost* 1984a; 52: 167–171.
81 Karpatkin S. Heterogeneity of human platelets. II. Functional evidence suggestive of young and old platelets. *J Clin Invest* 1969b; 48: 1083–1087.
82 Trowbridge EA, Martin JF. The platelet volume distribution: a signature of the prethrombotic state in coronary heart disease? *Thromb Haemost* 1987; 58: 714–717.
83 Martin JF, Bath PM, Burr ML. Influence of platelet size on outcome after myocardial infarction. *Lancet* 1991; 338: 1409–1411.
84 Metcalfe BC, Warren CW, Slater DN, Trowbridge EA, Martin JF, Barer GF. Changes in platelets and megakaryocyte in simulated high altitude. *Clin Sci* 1984; 6: 76P(Abstract).
85 Milner PC, Martin JF. Shortened bleeding time in acute myocardial infarction and its relation to platelet mass. *Br Med J (Clin. Res Ed)* 1985; 290: 1767–1770.
86 van der LB, Martin JF. A role for changes in platelet production

Cambridge Books Online © Cambridge University Press, 2009

in the cause of acute coronary syndromes. *Arterioscler Thromb Vasc Biol* 1999; 19: 672–679.
87 Odell TT, Murphy JR, Jackson CW. Stimulation of megakaryocytopoiesis by acute thrombocytopenia in rats. *Blood* 1976; 48: 211–229.
88 Mathur A, Robinson MSC, Cotton J, Martin JF, Erusalimsky JF. Platelet reactivity in acute coronary syndromes: evidence for differences in platelet behaviour between unstable angina and myocardial infarction. *Thromb Haemost* 2001; 85(6): 989–994.
89 Pizzulli L, Yang A, Martin JF, Luderitz B. Changes in platelet size and count in unstable angina compared to stable angina or non-cardiac chest pain. *Eur Heart J* 1998; 19: 80–84.
90 Kristensen SD, Bath PM, Martin JF. Differences in bleeding time, aspirin sensitivity and adrenaline between acute myocardial infarction and unstable angina. *Cardiovasc Res* 1990; 24: 19–23.
91 Trowbridge EA, Warren CW, Martin JF. Platelet volume heterogeneity in acute thrombocytopenia. *Clin Phys Physiol Meas* 1986; 7: 203–210.
92 Martin JF, Daniel TD, Trowbridge EA. Acute and chronic changes in platelet volume and count after cardiopulmonary bypass induced thrombocytopenia in man. *Thromb Haemost* 1987; 57: 55–58.
93 Cerutti A, Custodi P, Duranti M, Cazzola M, Balduini CL. Circulating thrombopoietin in reactive conditions behaves like an acute phase reactant. *Clin Lab Haematol* 1999; 21: 271–275.

Cambridge Books Online © Cambridge University Press, 2009

5

Platelet membrane proteins as adhesion receptors

Yoshiaki Tomiyama[1], Masamichi Shiraga[1], and Sanford J. Shattil[2]

[1]Department of Internal Medicine and Molecular Science, Graduate School of Medicine, Osaka University, Japan

[2]Department of Vascular Biology, The Scripps Research Institute, La Jolla, USA

Platelets play a crucial role not only in the formation of a normal hemostatic plug but also in formation of a pathologic thrombus, particularly within arteries subjected to high shear stress. Platelets circulate passively as they traverse a vascular tree lined by an intact monolayer of endothelial cells. As an initial step in hemostasis and thrombogenesis, platelets adhere to altered vascular surfaces or exposed subendothelial matrices. Following adhesion, they become activated, change shape, secrete granule contents, and aggregate to each other to form a primary hemostatic plug and to provide a catalytic surface to enhance blood coagulation. Thus, thrombus formation depends primarily on platelet adhesive interactions with both extracellular matrix proteins [e.g. von Willebrand factor (vWF), collagen, fibronectin] and cellular surfaces (e.g. platelets, leukocytes). In order to mediate these adhesive events, platelets possess numerous receptors specialized for this task. In this chapter, we will focus on the structure and function of platelet plasma membrane glycoproteins that serve as important adhesion receptors. One theme that emerges is that many, if not all, adhesion receptors also function as signalling receptors. Other chapters in this book will focus on surface membrane proteins that function primarily as receptors for excitatory and inhibitory agonists and on granule proteins, such as P-selectin and CD40 ligand, that may mediate adhesive functions concomitant with granule secretion (Chapters 9–13).

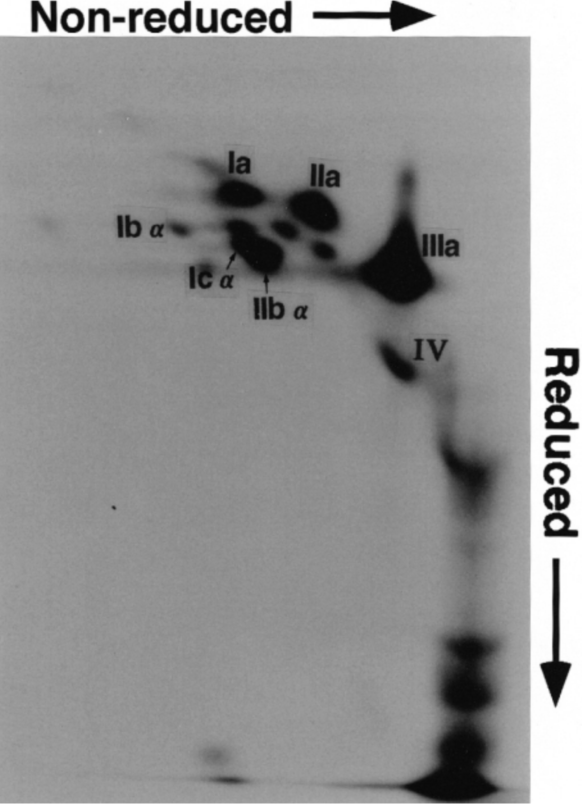


Fig. 5.1. Two-dimensional non-reduced–reduced SDS–polyacrylamide gel electrophoresis of surface-labelled intact platelets. ^{125}I-labelled platelets were electrophoresed in 5% polyacrylamide gels under non-reducing conditions in the first dimension. The gels were then electrophoresed in 7% polyacrylamide gels under reducing conditions in the second dimension. An autoradiograph of a dried gel is illustrated.

Platelet adhesion receptors

Surface labelling of intact platelets has shown that they possess numerous glycoproteins on their surface (Fig. 5.1)[1]. Initially, these glycoproteins were designated Ia, Ib,

Table 5.1. Platelet membrane glycoproteins that function as adhesion receptors

	Ligands	Platelet function	Molecules/platelet
Integrins			
β_1 integrins			
GPIa–IIa ($\alpha_2\beta_1$)	Collagen	Adhesion	<1000
GPIc–IIa ($\alpha_5\beta_1$)	Fn	Adhesion	<1000
GPIc′–IIa ($\alpha_6\beta_1$)	Laminin	Adhesion	<1000
β_3 integrins			
GPIIb–IIIa ($\alpha_{IIb}\beta_3$)	Fg/vWF/Fn/Vn	Aggregation/firm adhesion	~80 000
$\alpha_V\beta_3$	Fg/Vn/OP	?	50–100
Leucine-rich motif (LRK) family			
GPIb–IX	vWF/thrombin	Initial adhesion	~25 000
GPV			~12 000
GPIV (CD36)	TSP/collagen	Adhesion	12 000–19 000
GPVI	Collagen	Activation	~1000

Notes:
vWF: von Willebrand Factor, Fn: Fibronectin, Fg: Fibrinogen, Vn: Vitronectin, TSP: Thrombospondin, OP: Osteopontin.

Ic, IIa, IIb, IIIa, etc according to their apparent molecular sizes in SDS-polyacrylamide gels under non-reducing and reducing conditions. Among them, two major receptor complexes, GPIb–IX–V and GPIIb–IIIa, play essential roles in platelet adhesion and aggregation[2,3]. Platelet membrane glycoproteins have been cloned, sequenced and classified within known gene families (Table 5.1).

Platelet integrins

Integrins are a family of non-covalently associated $\alpha\beta$ heterodimers that mediate cellular attachment to the extracellular matrix and cell–cell cohesion[4,5]. To date, 18 different integrin α subunits and 8 different integrin β subunits have been defined, giving rise to more than 24 different integrins. They are often subdivided into groups based on integrin β subunits, such as β_1, β_2, and β_3 integrins[4,5]. Platelets possess at least five integrins ($\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$), and $\alpha_{IIb}\beta_3$ (GP IIb–IIIa) is the most abundant and functionally significant.

Structure

$\alpha_{IIb}\beta_3$ is a prototypic and platelet/megakaryocyte-specific integrin[6]. The α_{IIb} subunit has been found only in combination with β_3. $\alpha_{IIb}\beta_3$ is expressed on the platelet surface at a density of approximately 80 000 copies per cell[7]. Synthesis of α_{IIb} and β_3 is regulated by separate genes, both of which have been localized to chromosome 17q12.32[8,9]. However, the β_3 gene appears ≥365 kb upstream of the α_{IIb} gene[10]. α_{IIb} consists of a heavy chain (871 amino acid residues) containing four repeats of a putative divalent cation binding motif disulfide-linked to a light chain (137 amino acid residues). β_3 consists of a single polypeptide of 762 amino acids containing 56 cysteines which form extensive intramolecular disulfides (Fig. 5.2). The N-terminus and most of the remainder of each subunit are extracellular, and the membrane-spanning domain is connected to a short C-terminal cytoplasmic tail consisting of 20 amino acid residues in α_{IIb} and 47 residues in β_3. Electron microscopy and other analyses of heterodimers shows an N-terminal globular head connected to two C-terminal stalks[11–13]. Although the atomic structure of $\alpha_{IIb}\beta_3$ is not known, recent studies indicate that ligand binding is primarily a function of the globular heads[11–13].

Multiple binding sites for Arg–Gly–Asp (RGD)-containing ligands have been identified on both α and β subunits[14,15]. The N-terminal region of integrin α subunits has seven repeats of homologous sequences of about 60 amino acid residues. In several α subunits (e.g. α_2, α_L, α_M) but not α_{IIb}, an inserted domain of about 200 amino acid residues (the I-domain) is present between the second and the third repeats, which is critically involved in ligand binding[16,17], and its crystal structure has been determined[18,19]. This domain adopts a classic 'Rossmann' fold, with a central parallel β-sheet surrounded on both sides by α-helices and it contains a unique divalent cation coordination sphere, which has been designated as the metal ion-dependent adhesion site (MIDAS) motif. The conserved features of

Cambridge Books Online © Cambridge University Press, 2009

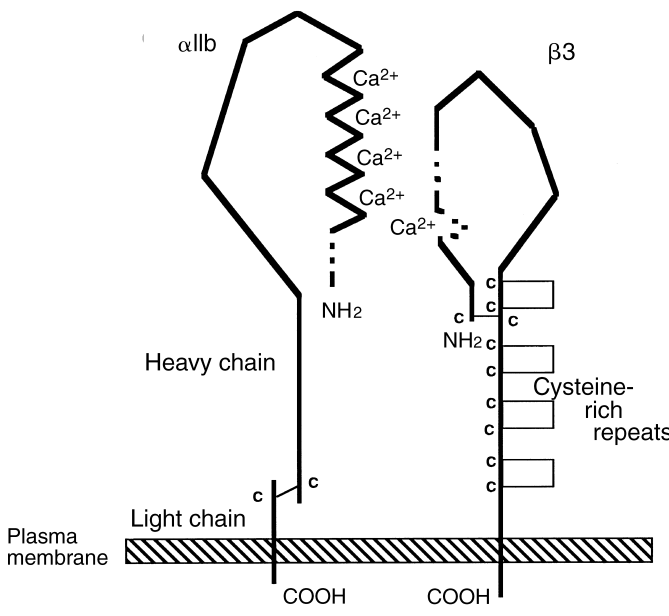


Fig. 5.2. Schematic representation of $\alpha_{IIb}\beta_3$. Dotted lines indicate putative ligand binding sites. α_{IIb} and β_3 are non-covalently associated at a stoichiometry of 1:1. α_{IIb} comprises a disulfide-linked heavy and light chain, while β_3 consists of a single polypeptide. Electron microscopic analyses of $\alpha_{IIb}\beta_3$ shows an N-terminal globular head connected to two C-terminal stalks.

this motif are an Asp X Ser X Ser (DXSXS) sequence, where X represents any amino acid. In the case of integrins like $\alpha_{IIb}\beta_3$ that do not contain the I domain in their α subunit. It has been proposed that β-subunits have an I-like domain located near the N terminus that contains an invariant DXSXS sequence (residues 119–123 in β_3). Using chemical cross-linkers, it has been shown that a ligand-mimetic RGD-containing peptide is cross-linked to β_3 residues 109–171[20]. A naturally occurring missense mutation at the first residue of DXSXS sequence (Asp119→Tyr) results in the inability of $\alpha_{IIb}\beta_3$ to recognize ligands, and alanine substitutions at these residues further support the hypothesis that this region of β_3 might engage ligands via a MIDAS-like motif[21,22].

Recently Springer proposed that the seven N-terminal sequence repeats of integrin α subunits are folded into a β-propeller domain, although there are as yet no high resolution structural data to support this model[23]. The proposed domains contain seven four-stranded β-sheets (W1–W7) arranged in a torus around a pseudosymmetry axis. Integrin ligands have been predicted to bind to the upper face of the β-propeller, especially within W2, W3 and W4[24,25]. Using chemical cross-linkers, it has been shown that a dodecapeptide derived from the C-terminus of the fibrinogen γ-chain is proximal to α_{IIb} residues 294–314, which contain the second putative calcium binding domain[26]. However, a recombinant α_{IIb} fragment comprising residues 1–233, which has no calcium-binding domains, can form a heterodimer with a recombinant β_3 fragment (residues 111–318) and bind to an RGD-containing peptide[27]. The residues 294–314 lie on the lower surface of the propeller far from the ligand-binding sites and may play a structural rather than ligand-binding role.

Function

$\alpha_{IIb}\beta_3$ recognizes several RGD-containing adhesive proteins in a divalent cation-dependent manner. In particular, the interaction with fibrinogen and vWF is crucial for platelet aggregation[28]. The ligand binding function of $\alpha_{IIb}\beta_3$ is dynamically regulated (Fig. 5.3)[29,30]. In resting platelets, $\alpha_{IIb}\beta_3$ is in a low affinity state and cannot bind soluble macromolecular ligands like fibrinogen or vWF. After platelet activation by agonists, intracellular signals are initiated that induce a high affinity/avidity state of $\alpha_{IIb}\beta_3$ with respect to these adhesive ligands. This process is frequently referred to as 'inside-out' signalling or 'integrin activation', and it encompasses at least two events: (i) modulation of receptor affinity through conformational changes and (ii) modulation of receptor avidity through facilitation of lateral diffusion and/or clustering of integrin heterodimers into oligomers. In the case of $\alpha_{IIb}\beta_3$, affinity modulation rather than avidity modulation seems to play the dominant role in initiating ligand-binding[30]. Based on mutational data, a model for the conformational switch in $\alpha_{IIb}\beta_3$ during platelet activation has been proposed[14]. Fibrinogen binding to $\alpha_{IIb}\beta_3$ itself alters the conformation of the integrin, which exposes neo-epitopes in the receptor known as ligand-induced binding sites (LIBS), and leads to 'post-receptor occupancy' events, including cytoskeletal reorganization[31–33]. Thus, $\alpha_{IIb}\beta_3$ and other integrins function as important signalling molecules that can mediate the bidirectional transfer of information across the platelet plasma membrane.

Evidence directly implicating the cytoplasmic tails in affinity modulation comes from studies of naturally occurring and experimental integrin mutations, from analyses of $\alpha_{IIb}\beta_3$ function in heterologous expression systems, and from identification of integrin tail-binding proteins[34–39]. Affinity modulation probably involves the propagation of a conformational change from the $\alpha_{IIb}\beta_3$ cytoplasmic domains to the extracellular domains. Concerning the potential structure of the α_{IIb} and β_3 cytoplasmic domains,

Cambridge Books Online © Cambridge University Press, 2009

Fig. 5.3. $\alpha_{IIb}\beta_3$ signalling. Conformation and function of $\alpha_{IIb}\beta_3$ are dynamically regulated. After platelet activation intracellular signals are initiated that induce a high affinity/avidity state of $\alpha_{IIb}\beta_3$ (inside–out signals). Fibrinogen binding to $\alpha_{IIb}\beta_3$ leads to outside–in signals.

a model has been proposed in which the membrane-proximal portions of the tails interact when the integrin is in a low affinity state, possibly through a salt bridge to form a hinge. Certain membrane-proximal mutations or deletions are hypothesized to break this hinge, converting $\alpha_{IIb}\beta_3$ to a high-affinity state[34,35,40]. This model predicts close but dynamic interactions between the α_{IIb} and β_3 tails, and synthetic peptides derived from these tails have been shown to interact in vitro[41].

Recently, mice have been generated that express $\alpha_{IIb}\beta_3$ in which the two tyrosine residues in the β_3 cytoplasmic tail (Y747 and Y759) have been switched to phenylalanine[42]. These mice exhibit normal inside-out but impaired outside-in signalling, with defective aggregation and clot retraction. Outside-in signalling through $\alpha_{IIb}\beta_3$ involves stimulation of numerous interacting pathways that feature activation of protein tyrosine kinases (e.g. Syk, Src family, FAK) and tyrosine phosphorylation of numerous substrates in addition to the β_3 tail[30]. How these pathways regulate cytoskeletal reorganization and other postligand binding events is an active area of investigation.

Deficiency

The importance of integrin $\alpha_{IIb}\beta_3$ is evident by the pathogenic consequence of its heritable deficiency, Glanzmann thrombasthenia (GT)[43]. GT is an autosomal recessive bleeding disorder that is due to a quantitative and/or qualitative defect in $\alpha_{IIb}\beta_3$. Molecular analysis in GT as well as transfection studies have demonstrated that a molecular abnormality in the genes for either α_{IIb} or β_3 can lead to the deficiency of $\alpha_{IIb}\beta_3$ on platelet surface. Absent and/or abnormally spliced mRNA due to a nonsense mutation or frame shift are common in severe $\alpha_{IIb}\beta_3$ deficiency (type I GT), whereas missense mutations in either subunit are common in moderate $\alpha_{IIb}\beta_3$ deficiency. Although various missense mutations leading to the impaired expression of $\alpha_{IIb}\beta_3$ are reported, it has been demonstrated that relatively well-conserved residues flanking the calcium-binding domains of α_{IIb} are particularly critical for $\alpha_{IIb}\beta_3$ expression[44–47]. The bleeding diathesis in GT is characterized by mucocutaneous hemorrhage with easy bruising, menorrhagia, epistaxis, and gingival bleeding. Platelet aggregation is absent in response to ADP, epinephrine, collagen, or thrombin, while ristocetin-induced platelet aggregation is observed (Fig. 5.4). GT due to a dysfunctional rather than an absent $\alpha_{IIb}\beta_3$ complex is referred to as variant GT, and the characterization of these variants has helped to identify residues on the integrin necessary for ligand binding and for bidirectional signalling[21,48–50].

Of particular interest are the Ser752→Pro substitution and the Arg724→Ter substitution in the cytoplasmic domain of β_3, each of which is responsible for disruption of the coupling between inside-out signalling and $\alpha_{IIb}\beta_3$ activation[38,39]. These GT variants provide strong evidence that the cytoplasmic domain of β_3 is involved in inside-out signalling. In addition, these GT variant platelets exhibit impaired outside-in signalling, resulting in reduced fibrin clot retraction and decreased cell spreading and cytoskeletal reorganization on fibrinogen[39,51]. Thus, while these

Cambridge Books Online © Cambridge University Press, 2009

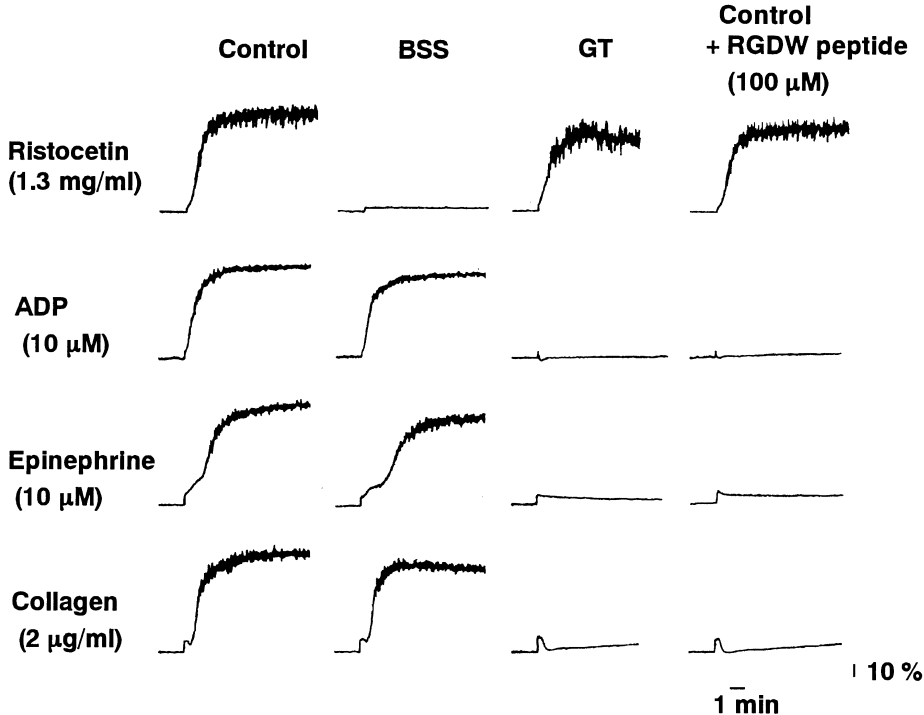


Fig. 5.4. Characteristics of impaired platelet aggregation observed in Bernard–Soulier syndrome (BSS) or Glanzmann thrombasthenia (GT). Ristocetin-induced agglutination depends on the interaction of von Willebrand factor (vWF) with GPIb–IX–V, while ADP-, epinephrine-, and collagen-induced platelet aggregation depend on the interaction of fibrinogen with $\alpha_{IIb}\beta_3$. Note that RGD-containing peptides induce the same defect in platelet aggregation observed in GT. These findings suggest that the interaction of fibrinogen with $\alpha_{IIb}\beta_3$ is mediated, at least in part, via the RGD sequence.

mutations in β_3 abrogate bidirectional transmembrane signalling via $\alpha_{IIb}\beta_3$, a β_3 Ser752→Ala mutation showed only minimal inhibitory effects on outside-in signalling when expressed in a CHO cell model system, suggesting that the presence of Pro752, rather than the absence of Ser752, is responsible for the phenotype[52,53].

Recently, β_3-deficient mice have been developed as an animal model for GT[54]. The β_3-null platelets show essentially the same defects as in human GT platelets. These mice show a greatly prolonged tail bleeding time and spontaneous hemorrhage. Moreover, they display additional defects that were not evident in studies of human GT, such as placental and bone resorption defects[54,55]. This animal model should prove to be a valuable tool for additional studies of GT pathogenesis and therapy.

Leucine-rich motif (LRM) family

The leucine-rich motif (LRM) family of proteins was first recognized as the result of a 24-residue motif present as 8 tandem copies in a human serum protein of unknown function called leucine-rich α_2-glycoprotein[56]. The LRM family, which is defined not by the similarity in the structure or function of its members, but by the presence of this structural motif, is a phylogenetically widespread family of proteins that is found in both eukaryotes and prokaryotes and as components of the nucleus, cytoplasm, cell membranes, extracellular matrix, and plasma. Four members of the LRM family, GPIbα, GPIbβ, GPIX and GPV, associate on the platelet surface to form the GPIb–IX–V complex[57,58]. This complex is the most prominent sialoglycoprotein in the platelet membrane and serves as a major receptor for vWF[58–60].

Structure

Each platelet contains approximately 25 000 copies of the GPIb–IX complex[61,62]. The four distinct transmembrane polypeptide subunits, GPIbα, GPIbβ, GPIX, and GPV, appear to be expressed at a stoichiometry of 2: 2: 2: 1, respectively (Fig. 5.5). A separate gene encodes each com-

Cambridge Books Online © Cambridge University Press, 2009

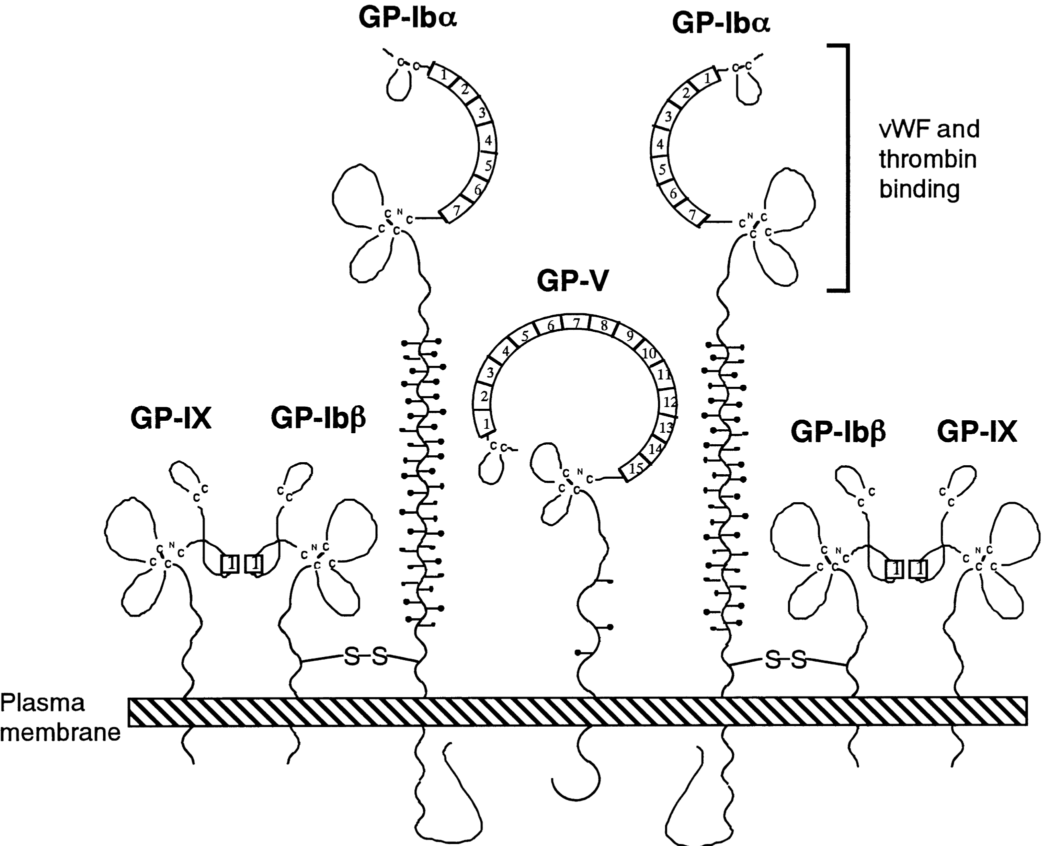


Fig. 5.5. Schematic representation of the GPIb–IX–V complex. Four members of the leucine-rich (LRM) motif family, GPIbα, GPIbβ, GPIX and GPV associate to form the GPIb-IX-V complex at a stoichiometry of 2:2:2:1. Box indicates leucine-rich repeat sequences (LRR), and GPIbα, GPIbβ, GPIX and GPV contain 7 tandem LRRs, one LRR, one LRR, and 15 LRRs, respectively. The N-terminal globular domain of GPIbα contains binding sites for von Willebrand factor (vWF) and thrombin. (Adapted from López et al., 1998 with permission.)

ponent of the GPIb–IX–V complex: the GPIbα, GPIbβ, GPIX, and GPV genes are located on chromosomes 17q12, 22q11.2, 3q21, and 3q29, respectively[63–66]. GPIb consisting of GPIbα (135 kDa, 610 amino acids) disulfide linked to GPIbβ (25 kDa, 181 amino acids) is noncovalently associated with GPIX (22 kDa, 160 amino acids). On electron microscopy, the purified GPIb–IX complex appears as a flexible rod with globular domains at either end and an overall length of 60 nm, which is 20–30 nm longer than $\alpha_{IIb}\beta_3$[67]. The smaller globular domain represents the N-terminal ligand-binding region of GPIbα, while the larger globular domain consists of the GPIbα transmembrane and cytoplasmic domains associated with GPIbβ and GPIX. The leucine-rich repeat sequences (LRR) are approximately 24 amino acids in length, and GPIbα, GPIbβ, GPIX and GPV contain 7 tandem LRRs, one LRR, one LRR, and 15 LRRs, respectively. The structure of GPIbα is divided into four distinct domains as follows: an N-terminal globular domain that contains ligand-binding sites, a mucin-like segment (macroglycopeptide), a transmembrane segment, and a cytoplasmic domain that interacts with intracellular proteins, such as filamin α and 14–3–3ζ[58–60].

Function

The major physiological role of the GPIb–IX–V complex is to mediate initial adhesion of circulating platelets to vWF in the subendothelial matrix under conditions of high shear. The transient and reversible platelet adhesion mediated by GPIb–IX–V then proceeds to irreversible platelet attachment mediated by the interaction of vWF with $\alpha_{IIb}\beta_3$[2,3]. The GPIb–IX–V complex interacts with vWF through a binding site in GPIbα located in the N-terminal 45-kDa domain (residues 1–293)[68,69]. The interaction between GPIb–IX–V and vWF appears to require a change in conformation in vWF and/or the GPIb–IX–V complex,

Cambridge Books Online © Cambridge University Press, 2009

induced either by the effects of shear on the ligand or receptor or by immobilization of vWF onto the subendothelial matrix. In the aggregometer, soluble vWF can support platelet–platelet interaction mediated through GPIb–IX–V using non-physiological modulators, such as ristocetin and the snake venom protein–botrocetin[58]. Although the vWF binding site has been localized to the 45-kDa domain of GPIbα, it has become evident that vWF binds to more than one site within this domain, and that binding is dependent upon the modulators by which the interaction is induced. One of the binding sites is the sulfated tyrosine sequence (residues 276, 278, 279) that is preferentially used for botrocetin-dependent vWF binding[70–72]. In addition, naturally occurring mutations have suggested that ligand-binding function requires the proper conformation of the 45 kDa domain[73,74]. Other potential roles for the GPIb–IX–V complex are to serve as a high affinity α-thrombin binding site, and as a receptor that participates in signal transduction to regulate the activation state of $\alpha_{IIb}\beta_3$[60,75].

Deficiency

The importance of the GPIb–IX–V complex is exemplified by the inherited bleeding disorder, Bernard–Soulier syndrome (BSS)[76]. BSS is an autosomal recessive disorder due to a quantitative and/or qualitative defect in the GPIb–IX–V complex. BSS is also characterized by a decrease in the platelet count with the striking phenotype of abnormally large or 'giant' platelets. A characteristic laboratory abnormality is an isolated defect in ristocetin-induced platelet agglutination (Fig. 5.4). In BSS the expression of all four subunits is impaired, however the defect in the expression of each subunit may not always be in the same proportion[77–79]. To date, molecular defects in BSS have been described in the GPIbα, GPIbβ and GPIX genes. However, no molecular defects have as yet been reported in the GPV gene[76]. As in GT, characterization of molecular defects leading to qualitative dysfunction of GPIb–IX–V has been informative. Two mutations leading to missense mutations within the extracellular LRR domain of GPIbα (Leu57→Phe and Ala156→Val) are associated with the impaired binding of vWF. These mutations reveal an important role of the LRR of GPIbα, which may function by maintaining the proper conformation and exposure of ligand-binding site[73,74]. In contrast, platelet-type von Willebrand disease is an autosomal-dominant bleeding disorder caused by mutations affecting the GPIb–IX–V complex, resulting in a gain-of-function phenotype[80,81]. The resultant mutants bind vWF with high affinity and the paradoxical presence of a bleeding diathesis is due to clearance of the hemostatically most active large vWF multimers. Two missense mutations (Gly233→Val and Met239→Val) within the Cys209–Cys248 loop of GPIbα are associated with a gain-of-function phenotype, suggesting that the loop may function as a regulatory region that prevents the exposure of the ligand-binding site[82–84].

The molecular basis for the giant platelets observed in BSS remains elusive. GPIbα-null mice also exhibit a bleeding diathesis as well as thrombocytopenia and giant platelets[85]. In addition, this mouse model of the BSS could be rescued with transgenic expression of a human GPIbα, demonstrating a direct link between expression of the GPIb–IX–V complex and platelet morphogenesis[85]. In contrast, platelets from GPV-null mice are normal in size and express normal amounts of GPIb–IX that is functional in vWF binding[86,87]. Thus, GPV is not required for the expression or function of GPIb–IX, which is consistent with the fact that mutations in GPV have not been observed in BSS.

With the impaired expression of GPIb–IX–V in BSS platelets, response to α-thrombin is also compromised[88]. Protease-activated G-protein-coupled receptors, PAR-1 and PAR-4, have a key role in the activation of human platelets by α-thrombin. It has been revealed that the 45-kDa domain of GPIbα that contains the vWF binding sites also is the region that binds thrombin[89]. Although the mechanism by which the binding of α-thrombin to GPIb contributes to platelet activation is obscure, a recent study suggests that binding of thrombin to GPIbα accelerates the hydrolysis of PAR-1 on the platelet membrane[90]. Thus, GPIb may function as a cofactor for PAR-1 activation by thrombin. In contrast to the normal vWF binding activity, platelets from GPV-null mice may be hyper-responsive to thrombin and GPV-null mice have a shorter than normal tail bleeding time[86]. These findings suggest a role for GPV in decreasing thrombin responsiveness of platelets, with experimental removal of GPV from the platelet surface contributing to platelet stimulation by thrombin.

CD36 (Glycoprotein IV)

Structure

CD36, also known as GP IV or GP IIIb, consists of 471 amino acid residues with N- and C-terminal hydrophobic portion domains that presumably represent transmembrane domains[91–93]. N- and C-terminal regions of CD36 are palmitoylated, further suggesting that both N- and C-terminal tails are cytoplasmic, and that the protein may partition to glycolipid-enriched membrane microdomains[94]. Because

Cambridge Books Online © Cambridge University Press, 2009

of its topology, most of the CD36 molecule is thought to be oriented to the extracellular milieu, and this region contains 10 potential N-glycosylation sites that likely accounts for this molecule being resistant to proteolysis[95]. CD36 is expressed in a wide variety of cell types, including platelets, monocytes, microvascular endothelial cells, mammary gland epithelial cells, activated keratinocytes, some melanoma cells. CD36 shares structural similarities with some molecules such as SR-B1/CLA-1 (a HDL receptor), LIMP-II (a lysosomal protein) and SNMP-1 (a membrane protein expressed in olfactory neurons), and these molecules are classified as the CD36 family[96–98].

Functions

CD36 is a multifunctional glycoprotein. From the viewpoint of platelet function, it has been shown to interact with a large variety of ligands such as collagen and thrombospondin[99,100]. However, CD36-deficient platelets bind to thrombospondin normally[101]. Although platelet aggregation by type V collagen was impaired, there have been conflicting reports as to the role of CD36 on platelet adhesion to collagen under static and flow conditions[102–105]. In contrast to the other platelet membrane protein deficiencies discussed above, CD36 deficiency is not associated with a bleeding diathesis. Thus, the role of CD36 in platelet function remains unclear. Beyond its role as a platelet glycoprotein, CD36 serves as a receptor on erythrocytes for the human malaria parasite, *Plasmodium falciparum*[91,106]. In addition, recent studies have suggested that CD36 plays an important role in lipoprotein and lipid metabolism[107–110]. CD36 has also been suggested as a candidate for an insulin-resistant gene[111], which is still a subject of controversy[112,113].

Deficiency

The first case of CD36 deficiency was found in a Japanese patient showing refractoriness to HLA-matched platelet transfusions due to an anti-Nak[a] isoantibody that recognizes CD36[114–116]. Platelet CD36 deficiency is divided into two subgroups according to expression of CD36 on monocytes: type I also lacks CD36 on monocytes and presumably on all other tissues, while type II expresses CD36 on monocytes[117,118]. Platelet CD36 deficiency is present in ~3% of the Japanese population and rare in Caucasians (~0.3%), and the majority of platelet CD36 deficiency is the type II phenotype[114,116]. Three genetic abnormalities have been identified, a missense mutation causing substitution of Pro[90] with Ser in CD36, a two-base deletion and a one-base insertion[119–121]. Careful clinical characterization of CD36 deficient subjects, especially type I subjects, would provide a better understanding of the role of CD36 in vivo.

Glycoprotein VI

It has been generally accepted that integrin $\alpha_2\beta_1$ (GPIa–IIa) and GPVI are major collagen receptors on platelets, playing critical roles in adhesion and activation, respectively[122,123]. The first indication that $\alpha_2\beta_1$ is a physiological collagen receptor came from the study of a patient whose platelets demonstrated decreased expression of GPIa and a failure to interact with collagen[124]. Similarly, the critical role for GPVI was first indicated by a patient with GPVI deficiency. Recently, it has become clear that GPVI plays an important role in platelet activation in response to collagen.

Structure

Recent molecular cloning of GP VI cDNA demonstrated that this receptor is a type I transmembrane protein belonging to the immunoglobulin (Ig) superfamily[125]. GPVI contains two Ig-C2 like extracellular domains formed by disulfide bonds and a 51 amino acid cytoplasmic tail.

Function

GP VI has been implicated in platelet–collagen adhesive interactions, together with integrin $\alpha_2\beta_1$[126,127]. GP VI exists in the platelet membrane in a non-covalent association with the FcRγ subunit, and GP VI surface expression is strictly dependent on its association with FcRγ[128]. Moreover, GP VI appears to function as the collagen-binding subunit, whereas FcRγ contains tandem immune receptor tyrosine-based activation motifs (ITAM) and functions as the signalling subunit of the complex. Collagen binding to GP VI-FcRγ induces platelet activation through signalling molecules such as Syk, SLP-76, LAT and phospholipase Cγ2[129,130]. Indeed, Syk becomes tyrosine phosphorylated upon collagen-induced platelet activation and associates with GP VI/FcRγ by binding to the ITAMs of FcRγ. Murine platelets deficient in FcRγ or Syk fail to become activated in response to collagen, although they do respond to other agonists[131].

Deficiency

The involvement of GPVI as a collagen receptor was first suggested in a Japanese patient with autoimmune thrombocytopenia whose platelets were unresponsive to

Cambridge Books Online © Cambridge University Press, 2009

collagen and defective in a 62 kDa platelet membrane protein, which was later identified as GPVI[132]. Three cases of GPVI-deficient patients without thrombocytopenia have been reported, and all of them have shown a mild bleeding tendency with a slightly prolonged bleeding time[122]. However, the molecular basis for GP VI deficiency has not yet been identified. GPVI-deficient platelets do not aggregate in response to collagen or adhere on immobilized collagen, whereas they do aggregate in response to other agonists. Mice injected with an anti-GP VI monoclonal antibody show rapid disappearance of GP VI from the platelet surface, reduced platelet responsiveness to GP VI agonists, and protection from lethal thrombogenic infusions of a mixture of collagen and epinephrine[133].

Conclusions

Numerous platelet membrane glycoproteins function as adhesion and signalling receptors and play a crucial role in hemostatic plug formation and pathological thrombus formation. Gene targeting and other experimental techniques in mice as well as naturally occurring quantitative or qualitative abnormalities of these receptors in humans have provided valuable information as to their functions in vivo. In addition, receptor–ligand interactions are beginning to be characterized at the molecular level. Thus, as exemplified by $\alpha_{IIb}\beta_3$, some platelet adhesion receptors are good targets for the development of new antithrombotic drugs[134,135]. Although not discussed here, other less characterized surface membrane glycoproteins, for example CD9 (a tetraspannin) and CD47 (integrin-associated protein), may also play roles in platelet adhesion and signalling, and their further characterization is warranted.

The crystal structure of the extracellular segment of integrin $\alpha_V\beta_3$ has recently been solved[136].

References

1 Phillips DR, Agin PP. Platelet plasma membrane glycoproteins: evidence for the presence of nonequivalent disulfide bonds using nonreduced-reduced two-dimensional gel electrophoresis. *J Biol Chem* 1977; 252: 2121–2126.
2 Savage B, Saldivar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell* 1996; 84: 289–297.
3 Goto S, Ikeda Y, Saldivar E, Ruggeri ZM. Distinct mechanism of platelet aggregation as a consequence of different shearing flow conditions. *J Clin Invest* 1998; 101: 479–486.
4 Hynes RO. Integrins: versatility, modulation, and signalling in cell adhesion. *Cell* 1992; 69: 11–25.
5 Ruoslahti E. Integrins. *J Clin Invest* 1991; 87: 1–5.
6 Phillips DR, Charo IF, Parise LV, Fitzgerald LA. The platelet membrane glycoprotein IIb-IIIa complex. *Blood* 1988; 71: 831–843.
7 Wagner CL, Mascelli MA, Neblock DS, Weisman HF, Coller BS, Jordan RE. Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets. *Blood* 1996; 88: 907–914.
8 Bray PF, Rosa JP, Johnston GI et al. Platelet glycoprotein IIb. Chromosomal localization and tissue expression. *J Clin Invest*, 1987; 80: 1812–1817.
9 Sosnoski DM, Emanuel BS, Hawkins AL et al. Chromosomal localization of the genes for the vitronectin and fibronectin receptors alpha subunits and for platelet glycoprotein IIb and IIIa. *J Clin Invest* 1988; 81: 1993–1998.
10 Thornton MA, Poncz M, Korostishevsky M et al. The human platelet α_{IIb} gene is not closely linked to its integrin partner β_3. *Blood* 1999; 94: 2039–2047.
11 Parise LV, Phillips DR. Reconstitution of the purified platelet fibrinogen receptor. Fibrinogen binding properties of the glycoprotein IIb–IIIa complex. *J Biol Chem* 1985; 260: 10698–106707.
12 Weisel JW, Nagaswami C, Vilaire G, Bennett JS. Examination of the platelet membrane glycoprotein IIb–IIIa complex and its interaction with fibrinogen and other ligands by electron microscopy. *J Biol Chem* 1992; 267: 16637–16643.
13 Hantgan RR, Paumi C, Rocco M, Weisel JW. Effects of ligand-mimetic peptides Arg–Gly–Asp-X (X = Phe, Trp, Ser) on αIIbβ3 integrin conformation and oligomerization. *Biochemistry* 1999; 38: 14461–14474.
14 Loftus, JC, Linddington RC. New insights into integrin–ligand interaction. *J Clin Invest* 1997; 99: 2302–2306.
15 Plow EF, Haas TA, Zhang Li, Loftus J, Smith JW. Ligand binding to integrins. *J Biol Chem* 2000; 275: 21785–21788.
16 Kamata T, Wright R, Takada Y. Critical threonine and aspartic acid residues within the I domains of β2 integrins for interactions with intercellular adhesion molecule 1 (ICAM-1) and C3bi. *J Biol Chem* 1995; 270: 12531–12535.
17 Michishita M, Videm V, Arnaout MA. A novel divalent cation-binding site in the A domain of the β2 integrin CR3 (CD11b/CD18) is essential for ligand binding. *Cell* 1993; 72: 857–867.
18 Lee J-O, Rieu P, Arnaout MA, Liddington R. Crystal structure of the A domain from the α subunit of integrin CR3 (CD11b/CD18). *Cell* 1995; 80: 631–638.
19 Qu A, Leahy DJ. Crystal structure of the I domain from the CD11a/CD18 (LFA, $\alpha_L\beta_2$) integrin. *Proc Natl Acad Sci USA* 1995; 92: 10277–10281.
20 D'Souza SE, Ginsberg MH, Burke TA, Lam SC-T, Plow EF. Localization of an Arg–Gly–Asp recognition site within an integrin adhesion receptor. *Science* 1988; 242: 91–93.
21 Loftus JC, O'Toole TE, Plow EF, Glass A, Frelinger AL III, Ginsberg MH. A β_3 integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. *Science* 1990; 249: 915–918.

Cambridge Books Online © Cambridge University Press, 2009

22 Bajt ML, Loftus JC. Mutation of a ligand binding domain of β_3 integrin. *J Biol Chem* 1994; 269: 20913–20919.
23 Springer TA. Folding of the N-terminal, ligand-binding region of integrin α-subunits into a β-propeller domain. *Proc Natl Acad Sci. USA* 1997; 94: 65–72.
24 Irie A, Kamata T, Puzon-McLaughlin W, Takada Y. Critical amino acid residues for ligand binding are clustered in a predicted β-turn of the third N-terminal repeat in the integrin α_4 and α_5 subunits. *EMBO J* 1995; 14: 5557–5568.
25 Irie A, Kamata T, Takada Y. Multiple loop structures critical for ligand binding of the integrin α_4 subunit in the upper face of the β-propeller model. *Proc Natl Acad Sci USA* 1997; 94: 7198–7203.
26 D'Souza SE, Ginsberg MH, Burke TA, Plow EF. The ligand binding site of the platelet integrin receptor GPIIb–IIIa is proximal to the second calcium binding domain of its α subunit. *J Biol Chem* 1990; 265: 3440–3446.
27 McKay BS, Annis DS, Honda S, Christie D, Kunicki TJ. Molecular requirements for assembly and function of a minimized human integrin $\alpha_{IIb}\beta_3$. *J Biol Chem* 1996; 271: 30544–30547.
28 Phillips DR, Charo IF, Scarborough RM. GPIIb–IIIa: the responsive integrin. *Cell* 1991; 65: 359–362.
29 Faull RJ, Ginsberg MH. Dynamic regulation of integrins. *Stem Cells* 1995; 13: 38–46.
30 Shattil SJ, Kashiwagi H, Pampori N. Integrin signalling. The platelet paradigm. *Blood* 1998; 91: 2645–2657.
31 Frelinger AL III, Lam SC, Plow EF, Smith MA, Loftus JC, Ginsberg MH. Occupancy of an adhesive glycoprotein receptor modulates expression of an antigenic site involved in cell adhesion. *J Biol Chem* 1988; 263: 12397–12402.
32 Frelinger AL III, Du X, Plow EF, Ginsberg MH. Monoclonal antibodies to ligand-occupied conformers of integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb–IIIa) alter receptor affinity, specificity, and function. *J Biol Chem* 1991; 266: 17106–17111.
33 Honda S, Tomiyama Y, Aoki T et al. Association between ligand-induced conformational changes of integrin $\alpha_{IIb}\beta_3$ and $\alpha_{IIb}\beta_3$-mediated intracellular Ca^{2+} signalling. *Blood* 1998; 92: 3675–3683.
34 O'Toole TE, Katagiri Y, Faull RJ et al. Integrin cytoplasmic domains mediate inside-out signal transduction. *J Cell Biol* 1994; 124: 1047–1059.
35 Hughes PE, O'Toole TE, Ylänne J, Shattil SJ, Ginsberg MH. The conserved membrane-proximal region of an integrin cytoplasmic domain specifies ligand binding affinity. *J Biol Chem* 1995; 270: 12411–12417.
36 Shattil SJ, O'Toole T, Eigenthaler M et al. β_3-endonexin, a novel polypeptide that interacts specifically with the cytoplasmic tail of the integrin β_3 subunit. *J Cell Biol* 1995; 131: 807–816.
37 Kashiwagi H, Schwartz MA, Eigenthaler M, Davis KA, Ginsberg MH, Shattil SJ. Affinity modulation of platelet integrin $\alpha_{IIb}\beta_3$ by β_3 endonexin, a selective binding partner of the β_3 integrin cytoplasmic tail. *J Cell Biol* 1997; 137: 1433–1443.
38 Chen Y-P, Djaffar I, Pidard D et al. Ser-752$\rightarrow$Pro mutation in the cytoplasmic domain of integrin β_3 subunit and defective activation of platelet integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb–IIIa) in a variant of Glanzmann thrombasthenia. *Proc Natl Acad Sci USA* 1992; 89: 10169–10173.
39 Wang R, Shattil SJ, Ambruso DR, Newman PJ. Truncation of the cytoplasmic domain of β_3 in a variant form of Glanzmann thrombasthenia abrogates signalling through the integrin $\alpha_{IIb}\beta_3$. *J Clin Invest* 1997; 100: 2393–2403.
40 Hughes PE, Diaz-Gonzalez F, Leong L et al. Breaking the integrin hinge: a defined structural constraint regulates integrin signalling. *J Biol Chem* 1996; 271: 6571–6574.
41 Haas TA, Plow EF. The cytoplasmic domain of $\alpha_{IIb}\beta_3$: a ternary complex of the integrin α and β subunits and a divalent cation. *J Biol Chem* 1996; 271: 6017–6026.
42 Law DA, DeGuzman FR, Heiser P, Ministri-Madrid K, Killeen N, Phillips DR. Integrin cytoplasmic tyrosine motif is required for outside-in $\alpha_{IIb}\beta_3$ signalling and platelet function. *Nature* 1999; 401: 808–811.
43 George JN, Caen JP, Nurden AT. Glanzmann's thrombasthenia: the spectrum of clinical disease. *Blood* 1990; 75: 1383–1395.
44 Coller BS, Seligsohn U, Peretz H, Newman PJ. Glanzmann thrombasthenia: new insights from an historical perspective. *Semin Hematol* 1994. 31: 301–311.
45 Bray PF. Inherited diseases of platelet glycoproteins: consideration for rapid molecular characterization. *Thromb Haemost* 1994; 72: 492–502.
46 French DL, Coller BS. Hematologically important mutations: Glanzmann thrombasthenia. *Blood Cell Mol Dis* 1997; 23: 39–51.
47 Tomiyama Y. Glanzmann thrombasthenia: integrin $a_{IIb}b_3$ deficiency. *Int J Hematol* 2000; 72: 448–454.
48 Bajt ML, Ginsberg MH, Frelinger ALIII, Berndt MC, Loftus JC. A spontaneous mutation of integrin $\alpha_{IIb}\beta_3$ (platelet glycoprotein IIb IIIa) helps define a ligand binding site. *J Biol Chem* 1992; 267: 3789–3794.
49 Lanza F, Stierle A, Fournier D et al. A new variant of Glanzmann's thrombasthenia (Strasbourg I): platelets with functionally defective glycoprotein IIb–IIIa complexes and a glycoprotein IIIa ^{214}Arg$\rightarrow$^{214}Trp mutation. *J Clin Invest* 1992; 89: 1995–2004.
50 Honda S, Tomiyama Y, Shiraga M et al. A two-amino acid insertion in the Cys146–Cys167 loop of the α_{IIb} subunit is associated with a variant of Glanzmann thrombasthenia. Critical role of Asp163 in ligand binding. *J Clin Invest* 1998; 102: 1183–1192.
51 Chen Y-P, O'Toole TE, Ylänne J, Rosa J-P, Ginsberg MH. A point mutation in the integrin β_3 cytoplasmic domain ($S^{752}\rightarrow P$) impairs bidirectional signalling through $\alpha_{IIb}\beta_3$ (platelet glycoprotein IIb–IIIa). *Blood* 1994; 84: 1857–1865.
52 Ylänne J, Huuskonen J, O'Toole TE, Ginsberg MH, Virtanen I, Gahmberg CG. Mutation of the cytoplasmic domain of the integrin β_3 subunit. Differential effects on cell spreading, recruitment to adhesion plaques, endocytosis, and phagocytosis. *J Biol Chem* 1995; 270: 9550–9557.

Cambridge Books Online © Cambridge University Press, 2009

53 Kieffer N, Melchior C, Guinet JM, Michels S, Gouon V, Bron N. Serine 752 in the cytoplasmic domain of the β_3 integrin subunit is not required for $\alpha_v\beta_3$ postreceptor signalling events. *Cell Adhes Commun* 1996; 4: 25–39.

54 Hodivala-Dilke KM, McHugh KP, Tsakiris DA et al. β_3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. *J Clin Invest* 1999; 103: 229–238.

55 McHugh KP, Hodivala-Dilke K, Zheng MH et al. Mice lacking β_3 integrins are osteosclerotic because of dysfunctional osteoclasts. *J Clin Invest* 2000; 105: 433–440.

56 Takahashi N, Takahashi Y, Putnam FW. Periodicity of leucine and tandem repetition of a 24-amino acid segment in the primary structure of leucine-rich α_2-glycoprotein of human serum. *Proc Natl Acad Sci USA* 1985; 82: 1906–1910.

57 Roth GJ. Developing relationships: arterial platelet adhesion, glycoprotein Ib, and leucine-rich glycoproteins. *Blood* 1991; 77: 5–19.

58 López JA. The platelet glycoprotein Ib–IX complex. *Blood Coagul Fibrinolysis* 1994; 5: 97–119.

59 Ware J. Molecular analyses of the platelet glycoprotein Ib–IX–V receptor. *Thromb Haemost* 1998; 79: 466–478.

60 Andrews RK, Shen Y, Gardiner EE, Dong JF, López JA, Berndt MC. The glycoprotein Ib–IX–V complex in platelet adhesion and signalling. *Thromb Haemost* 1999; 82: 357–364.

61 Berndt MC, Gregory C, Kabral A, Zola H, Fournier D, Castaldi PA. Purification and preliminary characterization of the glycoprotein Ib complex in the human platelet membrane. *Eur J Biochem* 1985; 151: 637–649.

62 Modderman PW, Admiraal LG, Sonnenberg A, von dem Borne AEGK. Glycoproteins V and Ib–IX form a noncovalent complex in the platelet membrane. *J Biol Chem* 1992; 267: 364–369.

63 Wenger RH, Wicki AN, Kieffer N, Adolph S, Hameister H, Clemetson KJ. The 5′ flanking region and chromosomal localization of the gene encoding human platelet membrane glycoprotein Ibα. *Gene* 1989; 85: 517–524.

64 Yagi M, Edelhoff S, Disteche CM, Roth GJ. Structural characterization and chromosomal location of the gene encoding human platelet glycoprotein Ibβ. *J Biol Chem* 1994; 269: 17424–17427.

65 Kelly MD, Essex DW, Shapiro SS et al. Complementary DNA cloning of the alternatively expressed endothelial cell glycoprotein Ibβ (GPIbβ) and localization of the GPIbβ gene to chromosome 22. *J Clin Invest* 1994; 93: 2417–2424.

66 Yagi M, Edelhoff S, Disteche CM, Roth GJ. Human platelet glycoproteins V and IX: mapping of two leucine-rich glycoprotein genes to chromosome 3 and analysis of structures. *Biochemistry* 1995; 34: 16132–16137.

67 Fox JE, Aggerbeck LP, Berndt MC. Structure of the glycoprotein Ib–IX complex from platelet membranes. *J Biol Chem* 1988; 263: 4882–4890.

68 Handa M, Titani K, Holland LZ, Roberts JR, Ruggeri ZM. The von Willebrand factor-binding domain of platelet membrane glycoprotein Ib. Characterization by monoclonal antibodies and partial amino acid sequence analysis of proteolytic fragments. *J Biol Chem* 1986; 261: 12579–12585.

69 Vicente V, Kostel PJ, Ruggeri ZM. Isolation and functional characterization of the von Willebrand factor-binding domain located between residues His1–Arg293 of the alpha-chain of glycoprotein Ib. *J Biol Chem* 1988; 263: 18473–18479.

70 Murata M, Ware J, Ruggeri ZM. Site-directed mutagenesis of a soluble recombinant fragment of platelet glycoprotein Ibα demonstrating negatively charged residues involved in von Willebrand factor binding. *J Biol Chem* 1991; 266: 15474–15480.

71 Dong JF, Li CQ, Lopez JA. Tyrosine sulfation of the glycoprotein Ib–IX complex: identification of sulfated residues and effect on ligand binding. *Biochemistry* 1994; 33: 13946–13953.

72 Marchese P, Murata M, Mazzucato M et al. Identification of three tyrosine residues of glycoprotein Ibα with distinct roles in von Willebrand factor and α-thrombin binding. *J Biol Chem* 1995; 270: 9571–9578.

73 Miller JL, Lyle VA, Cunningham D. Mutation of leucine-57 to phenylalanine in a platelet glycoprotein Ibα leucine tandem repeat occurring in patients with an autosomal dominant variant of Bernard–Soulier disease. *Blood* 1992; 79: 439–446.

74 Ware J, Russell SR, Marchese P et al. Point mutation in a leucine-rich repeat of platelet glycoprotein Ibα resulting in the Bernard–Soulier syndrome. *J Clin Invest* 1993; 92: 1213–1220.

75 Yap CL, Hughan SC, Cranmer SL et al. Synergistic adhesive interactions and signalling mechanisms operating between platelet glycoprotein Ib/IX and integrin $\alpha_{IIb}\beta_3$: studies in human platelets and transfected Chinese hamster ovary cells. *J Biol Chem* 2000; 275: 41377–41388.

76 López JA, Andrews RK, Afshar-Kharghan V, Berndt MC. Bernard–Soulier syndrome. *Blood* 1998; 91: 4397–4418.

77 Noda M, Fujimura K, Takafuta T et al. Heterogeneous expression of glycoprotein Ib, IX and V in platelets from two patients with Bernard–Soulier syndrome caused by different genetic abnormalities. *Thromb Haemost* 1995; 74: 1411–1415.

78 Wright SD, Michaelides K, Johnson DJ, West NC, Tuddenham EG. Double heterozygosity for mutations in the platelet glycoprotein IX gene in three siblings with Bernard–Soulier syndrome. *Blood* 1993; 81: 2339–2347.

79 Holmberg L, Karpman D, Nilsson I, Olofsson T. Bernard–Soulier syndrome Karlstad. Trp 498$\rightarrow$Stop mutation resulting in a truncated glycoprotein Ibα that contains part of the transmembranous domain. *Br J Haematol* 1997; 98: 57–63.

80 Miller JL, Castella A. Platelet-type von Willebrand's disease: characterization of a new bleeding disorder. *Blood* 1982; 60: 790–794.

81 Miller JL. Platelet-type von Willebrand disease. *Thromb Haemost* 1996; 75: 865–869.

82 Miller JL, Cunningham D, Lyle VA, Finch CN. Mutation in the gene encoding the alpha chain of platelet glycoprotein Ib in platelet-type von Willebrand disease. *Proc Natl Acad Sci USA* 1991; 88: 4761–4765.

Cambridge Books Online © Cambridge University Press, 2009

83 Russell SD, Roth GJ. Pseudo-von Willebrand disease: a mutation in the platelet glycoprotein Ib alpha gene associated with a hyperactive surface receptor. *Blood* 1993; 81: 1787–1791.
84 Takahashi H, Murata M, Moriki T et al. Substitution of Val for Met at residue 239 of platelet glycoprotein Ib alpha in Japanese patients with platelet-type von Willebrand disease. *Blood* 1995; 85: 727–733.
85 Ware J, Russell S, Ruggeri ZM. Generation and rescue of a murine model of platelet dysfunction: the Bernard–Soulier syndrome. *Proc Natl Acad Sci USA* 2000; 97: 2803–2808.
86 Ramakrishnan V, Reeves PS, DeGuzman F et al. Increased thrombin responsiveness in platelets from mice lacking glycoprotein V. *Proc Natl Acad Sci USA* 1999; 96: 13336–13341.
87 Kahn ML, Diacovo TG, Bainton DF, Lanza F, Trejo J, Coughlin SR. Glycoprotein V-deficient platelets have undiminished thrombin responsiveness and do not exhibit a Bernard–Soulier phenotype. *Blood* 1999; 94: 4112–4121.
88 Jamieson GA, Okumura T. Reduced thrombin binding and aggregation in Bernard–Soulier platelets. *J Clin Invest* 1978; 61: 861–864.
89 De Marco L, Mazzucato M, Masotti A, Ruggeri ZM. Localization and characterization of an α-thrombin-binding site on platelet glycoprotein Ibα. *J Biol Chem* 1994; 269: 6478–6484.
90 De Candia E, Hall SW, Rutella S, Landolfi R, Andrews RK, De Cristofaro R. Binding of thrombin to the Glycoprotein Ib accelerates the hydrolysis of Par-1 on intact platelets. *J Biol Chem* 2001; 276: 4692–4698.
91 Oquendo P, Hundt E, Lawler J, Seed B. CD36 directly mediates cytoadherence of *Plasmodium falciparum* parasized erythrocytes. *Cell* 1989; 58: 95–101.
92 Greenwalt DE, Lipsky RH, Ockenhouse CF, Ikeda H, Tandon NN, Jamieson GA. Membrane glycoprotein CD36: a review of its roles in adherence, signal transduction, and transfusion medicine. *Blood* 1992; 80: 1105–1115.
93 Daviet L, McGregor JL. Functional roles of membrane glycoprotein CD36. *Platelets* 1996; 7: 117–124.
94 Tao N, Wagner SJ, Lublin DM. CD36 is palmitoylated on both N- and C-terminal cytoplasmic tails. *J Biol Chem* 1996; 271: 22315–22320.
95 Nakata N, Furukawa K, Greenwalt DE, Sato T, Kobata A. Structural study of the sugar chains of CD36 purified from bovine mammary epithelial cells: occurrence of novel hybrid-type sugar chains containing the Neu5Ac alpha 2→6GalNAc beta 1→4GlcNAc and the Man alpha 1→2Man alpha→3Man alpha 1→6Man groups. *Biochemistry* 1993; 32: 4369–4383.
96 Vega MA, Segui-Real B, Garcia JA et al. Cloning, sequencing and expression of a cDNA encoding rat LIMP II, a novel 74-kDa lysosomal membrane protein related to the surface adhesion protein CD36. *J Biol Chem* 1991; 266: 16818–16824.
97 Calvo D, Vega MA. Identification, primary structure, and distribution of CLA-1, a novel member of the CD36/LIMP-II gene family. *J Biol Chem* 1993; 268: 18929–18935.
98 Rogers ME, Sun M, Lerner MR, Vogt RG. SNMP-1, a novel membrane protein of olfactory neurons of the silk moth *Antheraea polyphemus* with homology to the CD36 family of membrane proteins. *J Biol Chem* 1997; 272: 14792–14799.
99 Tandon NN, Kralisz U, Jamieson GA. Identification of GP IV (CD36) as a primary receptor for platelet-collagen adhesion. *J Biol Chem* 1989; 264: 7576–7583.
100 Asch AS, Barnwell J, Silverstein RL, Nachman RL. Isolation of the thrombospondin membrane receptor. *J Clin Invest* 1987; 79: 1054–1061.
101 Kehrel B, Kronenberg A, Schwippert B et al. Thrombospondin binds normally to glycoprotein IIIb deficient platelets. *Biochem Biophys Res Commun* 1991; 179: 985–991.
102 Kehrel B, Kronenberg A, Rauterberg J et al. Platelets deficient in glycoprotein IIIb aggregate normally to collagens type I and III but not to collagen type V. *Blood* 1993; 82: 3364–3370.
103 McKeown L, Vail M, Williams S, Kramer W, Hansmann K, Gralnick, H. Platelet adhesion to collagen in individuals lacking glycoprotein IV. *Blood* 1994; 83: 2866–2871.
104 Diaz-Ricart M, Tandon NN, Carretero M, Ordinas A, Bastida E, Jamieson GA. Platelet lacking functional CD36 (glycoprotein IV) show reduced adhesion to collagen in flowing whole blood. *Blood* 1993; 82: 491–496.
105 Saelman EUM, Kehrel B, Hese KM, de Groot PG, Sixma JJ, Nieuwenhuis HK. Platelet adhesion to collagen and endothelial cell matrix under flow conditions is not dependent on platelet glycoprotein IV. *Blood* 1994; 83: 3240–3244.
106 Ockenhouse CF, Tandon NN, Magowan C, Jamieson GA. Identification of a platelet membrane glycoprotein as a falciparum malaria sequestration receptor. *Science* 1989; 243: 1469–1471.
107 Endemann G, Stanton LW, Madden KS, Bryant CM, White RT, Protter AA. CD36 is a receptor for oxidized low density lipoprotein. *J Biol Chem* 1993; 268: 11811–11816.
108 Nozaki S, Kashiwagi H, Yamashita S et al. Reduced uptake of oxidized low density lipoproteins in monocyte-derived macrophages from CD36-deficient subjects. *J Clin Invest* 1995; 96: 1859–1865.
109 Abumrad NA, el-Maghrabi MR, Amri EZ, Lopez E, Grimaldi PA. Cloning of a rat adipocyte membrane protein implicated in binding or transport of long-chain fatty acids that is induced during preadipocyte differentiation. Homology with human CD36. *J Biol Chem* 1993; 268: 17665–17668.
110 Febbraio M, Abumrad NA, Hajjar DP et al. A null mutation in murine CD36 reveals an important role in fatty acid and lipoprotein metabolism. *J Biol Chem* 1999; 274: 19055–19062.
111 Aitman TJ, Glazier AM, Wallace CA et al. Identification of Cd36 (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats. *Nat Genet* 1999; 21: 76–83.
112 Gotoda T, Lizuka Y, Kato N et al. Absence of Cd36 mutation in the original spontaneously hypertensive rats with insulin resistance. *Nat Genet* 1999; 22: 226–228.
113 Pravenec M, Zidek V, Simakova M et al. Genetics of Cd36 and the clustering of multiple cardiovascular risk factors in spontaneous hypertension. *J Clin Invest* 1999; 103: 1651–1657.

Cambridge Books Online © Cambridge University Press, 2009

114 Ikeda H, Mitani T, Ohnuma M et al. A new platelet-specific antigen, Nak[a], involved in the refractoriness of HLA-matched platelet transfusion. *Vox Sang* 1989; 57: 213–217.
115 Tomiyama Y, Take H, Ikeda H et al. Identification of the platelet-specific alloantigen, Nak[a], on platelet membrane glycoprotein IV. *Blood* 1990; 75: 684–687.
116 Yamamoto N, Ikeda H, Tandon NN et al. A platelet membrane glycoprotein (GP) deficiency in healthy blood donors: Nak[a]-platelets lack detectable GPIV (CD36). *Blood* 1990; 76: 1698–1703.
117 Take H, Kashiwagi H, Tomiyama Y et al. Expression of GP IV and Nak[a] antigen on monocytes in Nak[a]-negative subjects whose platelets lack GP IV. *Br J Haematol* 1993; 84: 387–391.
118 Yamamoto N, Akamatsu N, Sakuraba H, Yamazaki H, Tanoue K. Platelet glycoprotein IV (CD36) deficiency is associated with the absence (type I) or the presence (type II) of glycoprotein IV on monocytes. *Blood* 1994; 83: 392–397.
119 Kashiwagi H, Tomiyama Y, Honda S et al. Molecular basis of CD36 deficiency. Evidence that a $^{478}C \rightarrow T$ substitution (proline90 $\rightarrow$ serine) in CD36 cDNA accounts for CD36 deficiency. *J Clin Invest* 1995; 95: 1040–1046.
120 Kashiwagi H, Tomiyama Y, Kosugi S et al. Identification of molecular defects in a subject with type I CD36 deficiency. *Blood* 1994; 83: 3545–3552.
121 Kashiwagi H, Tomiyama Y, Nozaki S et al. A single nucleotide insertion in codon 317 of the CD36 gene leads to CD36 deficiency. *Arterioscler Thromb Vasc Biol* 1996; 16: 1026–1032.
122 Moroi, M, Jung SM. Platelet receptors for collagen. *Thromb Haemost* 1997; 78: 439–444.
123 Watson SP. Collagen receptor signalling in platelets and megakaryocytes. *Thromb Haemost* 1999; 82: 365–376.
124 Nieuwenhuis HK, Akkerman JW, Houdijk WP, Sixma JJ. Human blood platelets showing no response to collagen fail to express surface glycoprotein Ia. *Nature* 1985; 318: 470–472.
125 Clemetson JM, Polgar J, Magnenat E, Wells TN, Clemetson KJ. The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcγR and the natural killer receptors. *J Biol Chem* 1999; 274: 29019–29024.
126 Moroi M, Jung SM, Okuma M, Shinmyozu K. A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion. *J Clin Invest* 1989; 84: 1440–1445.
127 Moroi M, Jung SM, Shinmyozu K, Tomiyama Y, Ordinas A, Diaz-Ricart M. Analysis of platelet adhesion to a collagen-coated surface under flow conditions: the involvement of glycoprotein VI in the platelet adhesion. *Blood* 1996; 68: 2081–2092.
128 Nieswandt B, Bergmeier W, Schulte V, Rackebrande K, Gessner JE, Zirngibl H. Expression and function of the mouse collagen receptor glycoprotein VI is strictly dependent on its association with the FcRγ chain. *J Biol Chem* 2000; 275: 23998–24002.
129 Ichinohe T, Takayama H, Ezumi Y, Yanagi S, Yamamura H, Okuma M. Cyclic AMP-insensitive activation of c-Src and Syk protein-tyrosine kinases through platelet membrane glycoprotein VI. *J Biol Chem* 1995; 270: 28029–28036.
130 Pasquet J, Gross B, Quek L et al. LAT is required for tyrosine phosphorylation of phospholipase Cγ2 and platelet activation by the collagen receptor GP VI. *Mol Cell Biol* 1999; 19: 8326–8334.
131 Poole A, Gibbins JM, Turner M et al. The Fc receptor γ-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *EMBO J* 1997; 16: 2333–2341.
132 Sugiyama T, Okuma M, Ushikubi F, Sensaki S, Kanaji K, Uchino H. A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia. *Blood* 1987; 69: 1712–1720.
133 Nieswandt B, Schulte V, Bergmeier W et al. Long-term antithrombotic protection by in vivo depletion of platelet glycoprotein VI in mice. *J Exp Med* 2001; 193: 459–469.
134 Coller BS. Platelet GPIIb/IIIa antagonists: the first anti-integrin receptor therapeutics. *J Clin Invest* 1997; 99: 1467–1470.
135 Topol EJ, Byzova TV, Plow EF. Platelet GPIIb–IIIa blockers. *Lancet* 1999; 353: 227–231.
136 Xiong JP, Stehle T, Diefenbach B et al. Crystal structure of the extracellular segment of integrin alpha Vbeta3. *Science* 2001; 294: 339–345.

Cambridge Books Online © Cambridge University Press, 2009

6

Dynamics of the platelet cytoskeleton

Kurt L. Barkalow, Hervé Falet and John Hartwig

Brigham & Women's Hospital, Hematology Division, LMRC Building, Rm 301, 221 Longwood Ave., Boston, MA O2115

Introduction

Platelets play a critical role in hemostasis and coagulation. At rest, they circulate in blood as small anucleated discs, measuring 3 × 0.5 µm. Following blood vessel injury and disruption of the endothelial layer, platelets avidly interact with exposed elements of the underlying connective tissue, a reaction that is further stimulated by soluble factor release. They rapidly change from discoid shapes into active forms, first by rounding, then by generating finger-like projections called filopodia and spreading over surfaces using thin sheet-like extensions called lamellipodia. A sturdy cytoskeleton composed of actin and tubulin polymers maintains the shape of the resting and activated platelet. Actin and tubulin are dynamic polymers that can be reversibly assembled. When assembled they can be crosslinked into higher-order structures such as bundles and networks, fragmented into smaller pieces, and slide relative to one another by motor proteins. A large cast of cytoskeletal-associated proteins controls these dynamic processes. Actin filament assembly, temporally and spatially, orchestrates the extension of filopodia and lamellipodia and shape transformation.

The cytoskeleton of the resting platelet

Resting platelets are discs (Fig. 6.1(a)) whose surfaces are smooth and featureless except for small membrane invaginations that mark entrances into the open canalicular system (OCS). Cytoskeletal proteins that maintain the discoid shape represent a large fraction of the platelet proteome. Actin, present at a concentration of 0.55 mM (230 000 actin subunits/platelet[1]), represents 20% of the total cellular protein. Actin subunits (called globular actin or G-actin) reversibly assemble into polymers (filaments of actin or F-actin). In the resting platelet, 40% of the total or ∼90 000 subunits of actin are assembled into 2000–5000 filaments of 0.5 to 1 µm lengths[1,2]. Actin filaments are polarized structures, first recognized by the way they interact with the myosin head domain, which bind periodically along the length of the filament to define pointed and barbed ends[3]. The barbed end of the filament is the preferred end in the assembly reaction, having the higher affinity for actin and grows at a rate ten times faster than the pointed end[4]. The barbed end of the filament is the only end that contributes to actin assembly in cells[5]. The G:F equilibrium of the resting cell is maintained by three mechanisms. First, virtually all (95–97%) of the actin filament barbed ends in the platelet are capped by high affinity binding proteins. Platelet proteins known to constitutively cap actin filament barbed ends are CapZ[6,7] and adducin, a protein that also caps the short actin filaments in the erythrocyte membrane skeleton[8,9]. Capping prohibits actin monomer addition to the barbed ends of filaments. Secondly, actin subunits not incorporated into filaments complex to proteins that prevent their addition to the pointed ends[10]. Proteins that bind actin monomers in this fashion are thymosin-β4[11] and profilin[12,13]. Both profilin and thymosin-β4 have higher affinities for G-actin than does the pointed filament end. Lastly, Arp2/3 complexes or other pointed end binding proteins may also cap many of the pointed ends in the platelet. Despite these many control mechanisms, actin filaments rapidly turnover in cells, particularly in regions of cells engaged in movement. The dynamic status of filaments in the platelet has not been directly investigated, but treatment with cytochalasins perturbs the G: F ratio, suggesting a flux of actin monomer between the two pools. Cofilin may participate in this process by

[1] $(0.55 \times 10^{-3}) \times (7 \times 10^{-15}) \times 6.023 \times 10^{23}$.

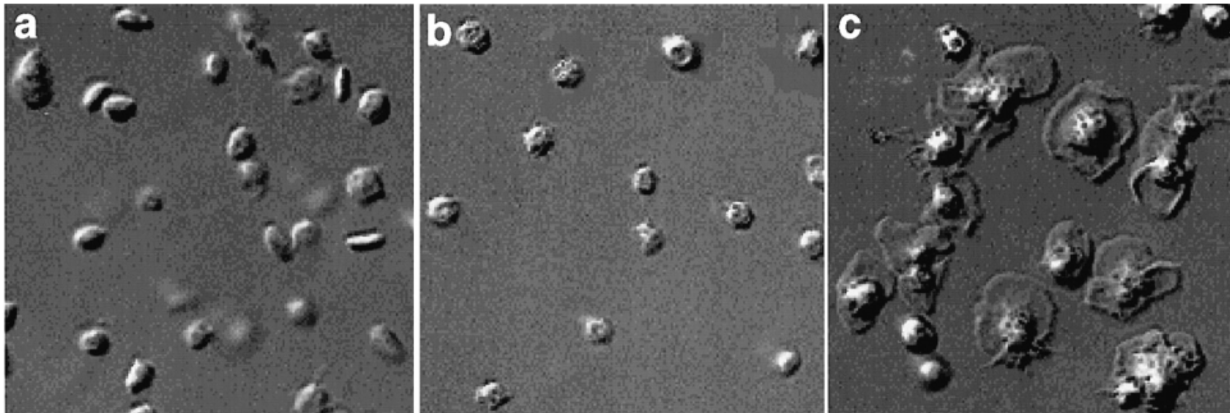


Fig. 6.1. Morphology of resting and activated platelets. Light micrographs, taken using differential interference contrast optics, showing (*a*) platelets at rest, (*b*) 10–15 s after activation of PAR-1 on platelets in suspension, and (*c*) platelets spread on glass for 10 min.

stimulating depolymerization from the pointed ends of filaments[14].

Actin filaments in the resting platelet are organized into a rigid cytoplasmic scaffold by filamin A (ABP-280), which can cross-link filaments at high angles, and by α-actinin, a protein that tends to align filaments in parallel. α-actinin is an abundant actin filament bundling protein that is often associated with adhesion sites in various cell types and has been shown to bind to the cytoplasmic tail of β1 integrins[15], suggesting a role in linking actin filaments to the membrane, in particular, at adhesion sites. Filamin A is a large homodimer whose 280 kD subunits self-associate at their carboxytermini while the amino-terminal domains contain actin binding sites, each capable of binding to a different actin filament. Filamin A also participates in actin–membrane interactions. The carboxyterminal portion of each subunit harbours a binding site for the integral membrane protein GP Ibα[16,17], a component of the von Willebrand factor receptor (vWFR) complex (GP Ib–IX–V)[18,19]. A stable link between the platelet plasma membrane and the cytoplasmic actin filaments is ensured by the complexing of $>$90% of the 25 000 copies of vWFR to filamin A[20]. The critical role of this interaction in maintaining the discoid shape has been established in studies of Bernard–Soulier Syndrome (BSS) platelets that lack the vWFR, and hence the vWFR–filamin–actin linkage. Platelets from BSS patients are abnormally large in size, fragile and circulate poorly, at least in part because their membrane skeleton (below) is weakly adhered to the underlying actin filaments.

A dense lattice of spectrin laminates the cytoplasmic side of the plasma membrane and is attached to the actin filament network of the resting platelet. Actin filament ends, derived from the filament network, selectively interact with the spectrin molecules. Capture of these ends by spectrin, which in theory should bind equally well along the length of the actin filament, may be related to the finding that many of the barbed ends in the resting platelet are capped by adducin. Adducin forms a high affinity ternary complex with spectrin and actin[21,22]. The near equivalent stoichiometry of actin filament ends (2 000–5 000), spectrin tetramers (2 000), and adducin tetramers in the resting cytoskeleton (5 000) allows for the possibility that all actin filaments which interconnect with the spectrin strands, do so via an adducin-capped barbed end. This idea is also consistent with observations that most of the actin filaments attach to the membrane skeleton by their barbed ends[1].

Traversing the pores of the spectrin network are the abundant filamin A linkages that connect vWFR to actin filaments. Actin filaments are linked at multiple points to the plasma membrane by filamin A as well as at their barbed ends by the spectrin-adducin interaction. This configuration imparts significant stability to the plasma membrane and to the discoid form of the platelet. Cleavage of these actin linkage proteins (filamin A and spectrin) by the protease calpain in aggregating platelets or during platelet storage, causes membrane blebbing and microvesicle release[23,24].

Tubulin and its associated proteins also contribute to the maintenance of the discoid shape of the resting platelet. Drugs that destabilize microtubules have been reported to cause the discoid shape in resting human platelets to be lost[25]. Approximately 40% of tubulin in a platelet is assembled into a specialized microtubule coil that lies just below the plasma membrane. This coil appears to form from a single microtubule that circumscribes the platelet periphery 8 to 12 times[26]. Microtubules do play an essential role

Cambridge Books Online © Cambridge University Press, 2009

in formation of platelets from megakaryocytes[27], although the precise details of how the microtubule coil develops to its mature form in the platelet are unknown. The microtubule-associated motors kinesin and cytoplasmic dynein are present in platelets[28]. Dynein localizes to punctate structures associated perhaps with intracellular organelles, but is surprisingly not enriched in the microtubule coil. A protein antigenically related to MAP2 has been shown to associate with the coil in resting bovine platelets[29].

The cytoskeleton of the activated platelet

Platelet shape change following receptor mediated activation proceeds through two recognizable steps (Fig. 6.1). First, the disc converts into an irregular or round shape (Fig. 6.1(*b*)). This transition requires a rise in intracellular calcium and activation of gelsolin to sever actin. Gelsolin severing in the cortex of the platelet destabilizes the highly ordered and constrained structure formed between spectrin/adducin- and filamin A-actin as discussed above[30]. With further surface contact, the platelet extends filopodia and spreads lamellae (Fig. 6.1(*c*)). Actin assembly drives these morphological changes.

Actin assembly occurs when actin monomers, stored in the cytoplasm and in complex with thymosin-β4 or profilin release and add to the barbed ends of actin filaments. Assembly of monomers into filaments is initiated by the exposure of nucleation sites which can be actin filament barbed ends or *de novo* nuclei. As discussed, proteins such as CapZ and adducin maintain >95% of the total actin filament barbed ends capped in resting platelets. Therefore, to trigger the actin assembly reaction either new barbed end nucleation sites must be generated de novo in the resting cell, or pre-existing and capped filaments must be uncapped. In platelets, both of these processes contribute in actin assembly. The changes seen in the actin cytoskeleton between resting and activated platelets are illustrated in the electron micrographs (Fig. 6.2). Detergent extraction of the resting platelet primarily shows the membrane skeleton, composed of spectrin and associated proteins as discussed above. Activation of the platelet results first in the loss of the discoid shape. The most striking cytoskeletal change during this ‘rounding’ is the appearance of short filaments in the cell cortex that derive from the severing of the preexisting cortical actin filaments. Spreading of the platelet requires actin assembly, and large lamellae become filled with branched networks of actin filaments. The template for this orthogonal filament array derives from fragmentation of actin filaments in the cortex of the

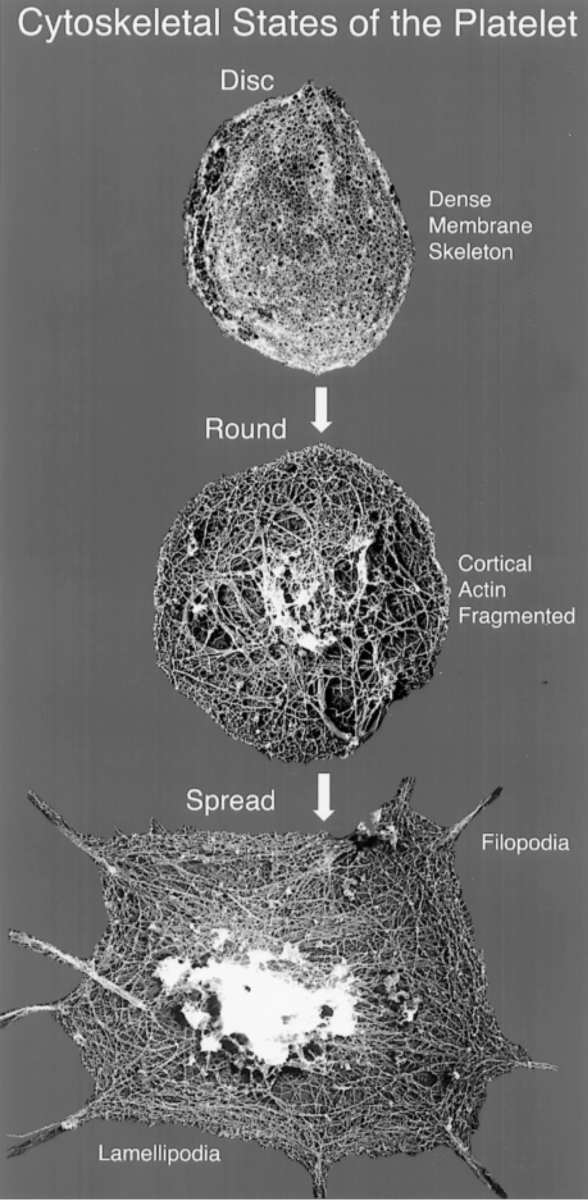


Fig. 6.2. Structure of the resting and active platelet cytoskeleton. Electron micrographs of platelet cytoskeletons at comparable stages to those shown in Fig. 6.1 (rest, round, spread). The cytoskeleton has been revealed by removing membrane with detergents. The fragmented actin filaments composing the rounded platelet cytoskeleton were captured by activating platelets in the presence of cytochalasin B, a reagent that blocks actin assembly.

Cambridge Books Online © Cambridge University Press, 2009

Platelet shape	Actin	Actin filament ends	Main signals
Discoid	40 % of actin polymerized into filaments	CapZ and adducin cap actin filament barbed ends	$[Ca^{2+}] < 100$ nM ppl's sequestered
Round	40 % of actin polymerized into filaments	Gelsolin severs actin filaments CapZ, adducin and gelsolin are on barbed ends	PLC-mediated hydrolysis of ppl's Cytosolic calcium rises to 0.5-3 μM
Spread	80 % of actin polymerized into filaments	Gelsolin and adducin dissociate from barbed ends Arp2/3 is activated and nucleates actin assembly de novo CapZ associates with barbed ends	ppl's are synthesized: PI_4P up by 80 μM $PI_{4,5}P_2$ up by 60 μM $PI_{3,4}P_2$ up >20 fold $PI_{3,4,5}P_3$ up transiently

Fig. 6.3 (see also colour plate). The role of calcium and ppIs in the regulation of the platelet actin cytoskeleton. Platelet shape change during activation is linked with changes in the actin cytoskeleton, proteins that regulate actin filament ends and the primary signals that effect changes in filament ends. The actin filaments in the discoid resting platelet are in a mustard-yellow colour, while the capping proteins are in pink and purple. Actin monomers that add to the barbed end of filaments in the spread platelet are illustrated as blue subunits.

platelet. Gelsolin-driven severing of actin and uncapping of these filaments occur in the cortex and ultrastructural studies demonstrate that this area supports new actin assembly[31]. Furthermore, filaments, after being severed, remain in the cortex since they are still linked to the plasma membrane by filamin A, and the polyphosphoinositides that mediate uncapping of gelsolin capped filaments reside in the plasma membrane. Figure 6.3 summarizes the changes in morphology, actin content, and critical regulatory proteins and signals that occur in the active platelet forms.

Changes in the microtubule cytoskeleton coincident with platelet activation have been described. The microtubule coil is either compressed into the center of the active platelet or rearranged to a number of individual microtubules that radiate out from the cell center into the cortex, particularly into filopodia. Whether this transition requires depolymerization and/or fragmentation of the coil is not known.

Regulation of actin assembly

There are at least four potential actin filament barbed end capping proteins present in platelets that can serve as targets for signals that lead to actin filament uncapping (Figs. 6.3 and 6.4, see colour plates). Gelsolin is one of the best-studied molecules in terms of its effect on actin in vitro[32,33]. Gelsolin, a major actin filament barbed end capping protein in platelets (5 μM), is also capable of severing actin filaments. Gelsolin is activated to bind to actin in μM calcium. In the resting platelet, the cytoplasmic calcium level is $<$100 nM, and gelsolin is not associated with actin[34]. Following ligation of the thrombin receptor (PAR-1), a rise in cytoplasmic calcium activates gelsolin to bind actin; $>$90% of platelet gelsolin is complexed with actin after 5 s[34]. Association with actin begins with its binding to the side of an actin filament, followed by interdigitation into the filament to sever it. After severing, gelsolin remains on the newly formed actin filament barbed end. Gelsolin-driven severing primarily takes place in the platelet cortex, and severing of these cortical actin filaments causes platelets to lose their discoid shape. Activation of gelsolin and actin–gelsolin complex formation occur before the actin assembly reaction begins. The initial complexing of gelsolin to actin is followed by a dramatic decrease in the amount of gelsolin bound to actin, and by 90 seconds after platelet activation, ~50% of the gelsolin complexed with actin in the first seconds of platelet activation is released. Gelsolin-actin dissociation is mediated by membrane phospholipids (polyphosphoinositides, ppIs[35], and lysophosphatidic acid, LPA[36]), whose synthesis after platelet activation is stimulated by small GTPase activation. The signal transduction cascade leading to new production of ppIs is described below. Mouse platelets from transgenic

Cambridge Books Online © Cambridge University Press, 2009

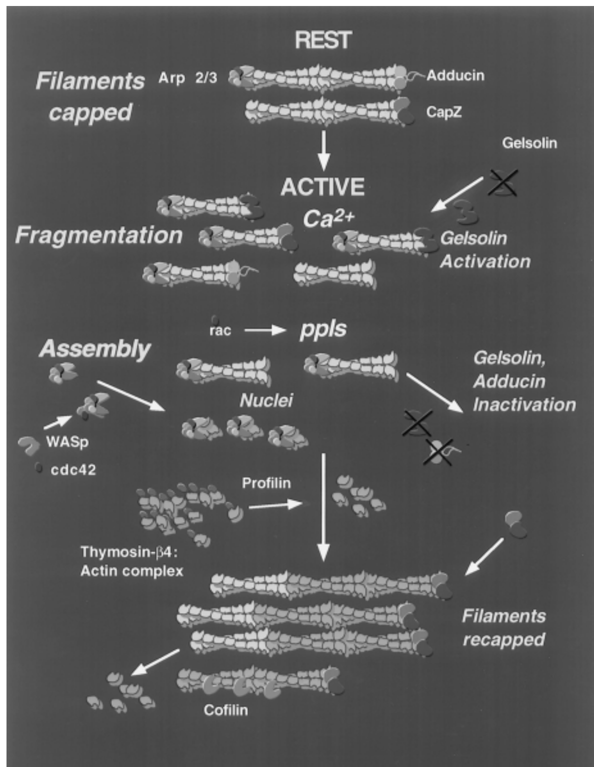


Fig. 6.4 (see also colour plate). Regulation of platelet actin assembly. The role of known platelet proteins in the actin assembly reaction is shown. In the resting platelet, adducin and CapZ cap actin filament barbed ends. Activation leads to calcium release and the activation of gelsolin. Gelsolin fragments the cortical actin filament network and this event is responsible for the loss of the discoid shape of the resting platelet, which now assumes a more rounded morphology. Polyphosphoinositides are robustly generated at the cytoplasmic surface of the platelet plasma membrane and lead to the initiation of actin assembly onto exposed barbed ends of actin filaments. PpIs both inactivate capping proteins (adducin and gelsolin) and maximally activate the Arp2/3 complex through the WASp family of proteins. Profilin and thymosin $\beta 4$, by binding to actin monomers, maintain the large actin pool that drives filament assembly. Actin filaments are subsequently buffered by CapZ, which recaps the filaments, and terminates the assembly reaction. Subsequent actin filament turnover if it occurs in platelets is stimulated by cofilin.

animals that lack gelsolin have a 50–75% reduction in their capacity to generate barbed end nucleation sites and assemble actin following platelet activation. These results demonstrate that gelsolin is directly responsible for much of the actin assembly events (exposure of actin filament barbed ends) during platelet activation.

Another important actin filament barbed end capping protein in platelets is CapZ[6,7]. Platelets have 5 μM CapZ, one third of which is associated with actin in resting platelets. With activation of platelets, more CapZ is deposited onto actin. The kinetics of CapZ association with actin closely follows the kinetics of actin assembly and binding reaches a maximum when actin assembly ceases. CapZ's association with actin, therefore, reaches a maximum after the gelsolin-actin interaction is reversed. This normal synergistic interaction of CapZ with gelsolin-generated ends is defective in gelsolin deficient platelets. Platelets from transgenic mice lacking gelsolin have more polymerized actin and consequently, the amount of CapZ associated with actin is elevated (~50% of the total being associated with the actin cytoskeleton). The amount of CapZ bound to actin following activation, however, does not change, indicating that CapZ incorporation into the actin cytoskeleton depends on the gelsolin-driven actin filament fragmentation reaction. This information shows that CapZ acts primarily as an actin assembly buffer to maintain actin filament barbed ends capped and inaccessible at rest and to terminate assembly in activated platelets by capturing exposed barbed ends. Although these experiments show that CapZ's main function is to buffer actin filament barbed ends, a small amount can be released from actin by signals generated by receptor ligation. In vitro, CapZ is released from actin filament barbed ends by polyphosphoinositides[37] and following treatment of permeabilized platelets with micelles of the polyphosphoinositides $PI_{3,4}P_2$ or $PI_{4,5}P_2$ or following PAR-1 ligation, a small amount of CapZ (~8%) releases from actin filament ends[6]. These data indicate that CapZ can be a target in signal transduction pathways.

Flightless-I, a gelsolin homologue, is also expressed in human platelets and associated with actin filaments comprising the cytoskeletons of resting and activated platelets. Flightless-I has an intriguing structure. In addition to having a domain that is homologous to the full amino acid sequence of gelsolin, it also contains an amino-terminal leucine rich repeat domain that binds other proteins[38]. Flightless-I is present at one-tenth of the concentration of CapZ or gelsolin. Therefore, although the specific details of its function have not been worked out, it may participate in cytoskeletal dynamics.

The last capping protein that has been studied in platelets is adducin. Adducin has been well described in erythrocytes where it caps the barbed end of small actin filaments composing the spectrin membrane skeleton. Platelets contain ~3 μM adducin and 80–85% of it is associated with the resting cytoskeleton. Several signalling molecules modulate adducin–actin interactions including calcium–calmodulin, phosphorylation by protein kinase C (PKC) or Rho-associated protein kinase (ROCK)[39,40], and

Cambridge Books Online © Cambridge University Press, 2009

possibly polyphosphoinositides by regulating the interaction of adducin with actin via the MARCKS domain in adducin[41,42]. Calcium–calmodulin, phosphorylation by PKC, and possibly polyphosphoinositides, are negative regulators of the adducin actin interaction, whereas phosphorylation by ROCK is a positive regulator. Unpublished data (K.L. Barkalow) suggest that α-adducin in platelets is largely associated with the actin cytoskeleton in resting platelets, but becomes soluble with platelet activation, coincident with phosphorylation by PKC. These data are consistent with a role for adducin in actin filament barbed end uncapping in early platelet activation.

De novo actin nucleation by WASp and the Arp2/3 complex

Recent evidence indicates that WASp family proteins integrate signals leading to nucleation of actin by the Arp2/3 complex[43,44]. WASp and Arp2/3 are likely to contribute to the burst of actin assembly that follows platelet activation. WASp is the protein mutated in Wiskott-Aldrich Syndrome (WAS), a rare X-linked recessive disorder characterized by eczema, severe immunodeficiency and thrombocytopenia[45]. The disease affects most hematopoietic lineages, including lymphocytes, monocytes, neutrophils and platelets. Platelets are among the most severely affected cells. Platelets from WAS patients, which lack WASp, are abnormally small, and circulating counts can be 10% of normal or lower. Other WASp family members have been identified. A gene encoding a protein highly homologous to WASp was cloned from bovine brain and was termed neural WASp (N-WASp)[46]. Unlike WASp, which is only expressed in hematopoietic cells, N-WASP is widely expressed. Subsequently, a novel protein related to WASp, WAVE/Scar, was identified, first in *Dictyostelium discoideum*, and then in vertebrates[47,48]. WASp family members interact with numerous signalling molecules known to alter the actin cytoskeleton. WASp contains an N-terminal pleckstrin-homology (PH) domain, which partially overlaps with a WASp-homology (WH) domain, WH1, found in Ena/VASP family proteins that are involved in the maintenance of cytoskeletal integrity[49]. C-terminal to this domain is a GTPase-binding domain (GBD), which binds the small GTPase Cdc42, a number of proline-rich stretches, a second WH domain, WH2, which has homology to the yeast protein verprolin, a cofilin-homology (CH) sequence and an acidic C-terminal region[50]. Overexpression of WASp in cultured cells leads to formation of actin clusters[49], and inducible recruitment of Cdc42 and WASp to a membrane receptor triggers actin polymerization that results in filopodia formation[51].

In the past few years, it became clear that WASp family proteins interact with the Arp2/3 complex to induce nucleation of actin filaments[43,44]. Arp2/3, originally found in *Acanthamoeba castellanii*, consists of two actin-related proteins, Arp2 and Arp3, and five novel proteins[52,53]. Arp2/3 nucleates actin filaments, but its activity requires WASp family proteins. Binding of Cdc42 and membrane ppIs to the WASp homologue N-WASp exposes the N-WASp C-terminus to Arp2/3 and actin monomers to mediate their association, initiate actin polymerization, and form new actin filaments in vitro[54]. It has been shown that Arp2/3 initiates the actin-based motility of intracellular parasites such as *Shigella flexneri* and *Listeria monocytogenes*[55–57]. Arp2/3 localizes to the leading edge of crawling cells such as *Xenopus laevis* keratocytes[58], suggesting that Arp2/3 is responsible for branching of actin filaments and de novo actin filament nucleation at the cell cortex that leads to cell movement.

The roles of WASp and Arp2/3 in platelet shape change and actin assembly are unclear. WASp is phosphorylated on tyrosine during platelet activation induced by collagen and a cross-linked collagen-related peptide specific for GPVI by a mechanism dependent on PI 3-kinase and actin assembly[59,60]. Arp2/3 localizes at the edge of the lamellipodia of platelets spreading on glass and redistributes to the actin cytoskeleton after platelet activation by the thrombin receptor PAR-1 and the collagen receptor GPVI (Falet, unpublished data). However, WASp-deficient platelets do not show any defect in actin assembly and shape change[60,61], and Arp2/3 redistributes normally to the actin cytoskeleton in the absence of WASp. These observations suggest that platelets differ from the other lymphoid cells affected by WASp deficiency and may recruit Arp2/3 to nucleate actin filaments in the absence of WASp by mechanisms that are yet to be discovered. Noteworthy, human platelets express WAVE/Scar, but not N-WASp[62] (Falet, unpublished data).

Signalling leading to platelet shape change

Platelet shape change and actin assembly are mediated by the production of two essential signalling molecules. First, cytosolic calcium concentrations are elevated by the release of intracellular stores, which requires the hydrolysis of plasma membrane polyphosphoinositides (ppIs) by phospholipase C (PLC), and by opening of calcium channels. The second key signal for platelet shape change is the production of ppIs downstream of the small GTPases[63].

Cambridge Books Online © Cambridge University Press, 2009

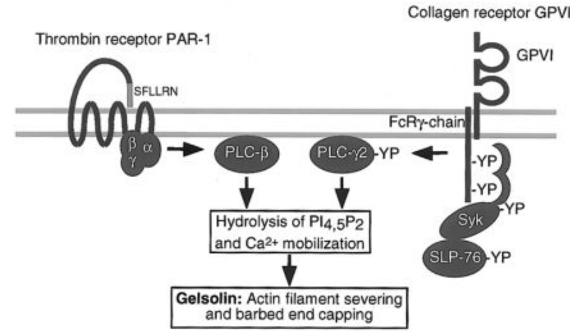
Cytosolic calcium increase

A rise in intracellular calcium results from the activation of PLC. Polyphosphoinositides (ppIs), notably phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5–P_2), are cleaved by PLC into inositol-1,4,5-trisphosphate (Ins-1,4,5–P_3) and diacylglycerol. The platelet calcium increase occurs through both Ins-1,4,5–P_3 mediated release of calcium from the intracellular stores and by the entry of calcium from the external medium. The diacylglycerol produced by this reaction goes on to activate protein kinase C (PKC)[64,65]. Thrombin and collagen, two of the most potent platelet activators, use distinct receptors, and their signalling pathways lead to the activation of PLC-β and PLC-γ2, respectively (Fig. 6.5).

Thrombin is a serine protease that plays a central role in hemostasis and coagulation. Thrombin binds to the vWFR and activates at least two protease-activated receptors (PAR) on human platelets: PAR-1 and PAR-4, which both belong to the seven-transmembrane receptor family that couples trimeric G-proteins. PAR-1 was the first PAR to be cloned, and its signalling pathway is well characterized[66,67]. Cleavage of its N-terminus by thrombin leads to its activation and the cytoplasmic stimulation of PLC-β through G-proteins of the Gq family[65]. Active PLC-β hydrolyzes PtdIns-4,5–P_2 in the plasma membrane as described above. PAR-1 ligation alone is sufficient to initiate platelet activation. Peptides having the exposed N-terminal SFFLRN sequence of cleaved PAR-1 mimic most platelet responses initiated by thrombin. These peptides, called thrombin receptor activating peptides (TRAP), are widely used in the literature as selective PAR-1 activators.

The platelet collagen receptor GPVI belongs to the immunoglobulin superfamily and forms a complex with the Fc receptor γ-chain (FcRγ-chain) that contains an immunoreceptor tyrosine-based activation motif (ITAM) and the two Src-family tyrosine kinases, Fyn and Lyn[68–71]. Platelet stimulation by collagen, or a cross-linked collagen-related peptide (CRP) selective for GPVI, significantly increases the tyrosine phosphorylation of multiple signalling proteins, including FcRγ-chain, the tyrosine kinase Syk, the adaptor proteins SLP-76 and LAT, and PLC-γ2. A model has been proposed where FcRγ-chain ITAM is phosphorylated by Fyn and Lyn after GPVI ligation and binds Syk through Syk tandem Src homology 2 (SH2) domains. SLP-76 is phosphorylated by Syk and recruits PLC-γ2 to the plasma membrane, where Syk phosphorylates and activates it. In support of this model, tyrosine phosphorylation of PLC-γ2 and platelet activation induced by collagen and CRP are abolished in FcRγ-chain-, Syk- and SLP-76-deficient mice[72–75]. It has also been shown that phospho-

1. Ca^{2+} mobilization and actin filament severing



2. Production of ppIs and actin assembly

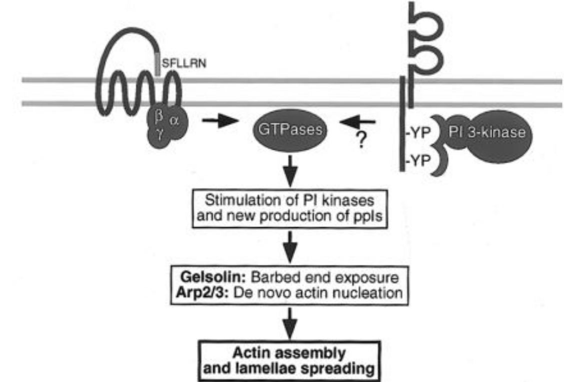


Fig. 6.5. Scheme for signal transduction from the thrombin receptor PAR-1 and the collagen receptor GPVI to platelet actin assembly and shape change. (*a*) Engagement of PAR-1 results in the GTP loading and the dissociation of Gqα from the trimeric G protein complex. Gqα-GTP activates PLC-β, leading to the hydrolysis of membrane ppIs. This mediates Ca^{2+} mobilization, resulting in the activation of gelsolin, which severs the actin filaments and caps their barbed ends. Engagement of GPVI activates the protein tyrosine kinase Syk, which phosphorylates the adaptor protein SLP-76. SLP-76 recruits PLC-γ2 to the plasma membrane, where Syk phosphorylates and activates it. This also results in Ca^{2+} mobilization and activation of gelsolin. (*b*) Subsequently, platelet activation also results in the activation of small Rho GTPases, which bind and activate PI kinases, leading to a new production of membrane ppIs. PpIs inactivate gelsolin and mediate the uncapping of the actin filament barbed ends; they also stimulate WASp family proteins, leading to the *de novo* nucleation of actin filaments by Arp2/3. Both PAR-1 and GPVI induce the actin assembly required for the formation of lamellae. Engagement of GPVI requires PI 3-kinase activation.

Cambridge Books Online © Cambridge University Press, 2009

inositide (PI) 3-kinase is involved in GPVI signalling. PI 3-kinase binds to the phosphorylated FcRγ-chain ITAM after GPVI ligation by collagen or CRP[76], and its inhibition by wortmannin or LY294002 affects the platelet responses downstream of GPVI[73,77,78]. Membrane ppIs phosphorylated in the D3 position of the inositol ring modulate PLC-γ2 activity by binding to its pleckstrin homology and/or SH2 domains[79,80]. Noteworthy, human platelets express another ITAM-containing receptor: the immunoglobulin G Fc receptor FcγRIIA. It is believed that GPVI and FcγRIIA share similar signalling pathways leading to PLC-γ2 activation and calcium mobilization[79,81].

Small GTPases and new production of membrane polyphosphoinositides

The key signal for platelet shape change is the new production of ppIs[63]. As described above, ppIs couple to multiple actin regulatory proteins. They inactivate capping proteins and are critical co-factors in the activation of WASp family proteins, which both lead to actin assembly. Synthesis of ppIs is driven by small GTPase activation of PI kinases.

Small GTPases of the Rho family have been shown to control actin assembly and architecture. The paradigm described first by Hall and collaborators, using microinjected or transfected fibroblasts, is that Cdc42 controls the formation of filopodia, Rac the extension of lamellipodia and Rho the formation of stress fibres and adhesion sites[82]. Rac binds to PI 5-kinase and stimulates its activity[83]. In permeabilized platelets, dominant negative Rac or kinase-dead PI 5-kinase block the signalling from PAR-1 to actin assembly[63]. Furthermore, a rapid and robust activation of Rac follows ligation of the thrombin receptor PAR-1 causing Rac to localize at the edge of the lamellipodia of platelets spreading on glass[84]. Activation of Rac correlates with the new production of ppIs observed after PAR-1 ligation. No redistribution to the actin cytoskeleton has been described in platelets activated in solution in non-aggregating conditions.

Small GTPases exist in two forms: an inactive GDP-bound form and an active GTP-bound form. Activation of Rho family GTPases requires guanine nucleotide exchange factors (GEF). However, the mechanisms of activation of these GTPases downstream of trimeric G-proteins are still unclear. In platelets, PAR-1 ligation leads to the dissociation of G_{12} family of trimeric G-proteins. The $G_{12}\alpha$ subunit, in other cells, has been shown to activate Rho GEFs. Other GEFs, e.g. Vav and Trio, may be important upstream mediators in the signalling to Rac and Cdc42. PI 3-kinase is also involved in the activation of Rac[85–87]. The lipid products of PI 3-kinase interact with Rac and stimulate GDP dissociation from Rac[88]. However, PI 3-kinase is not required for Rac activation, actin assembly or platelet spreading mediated by PAR-1[89]. PI 3-kinase is involved in platelet spreading over fibrinogen-coated surfaces mediated by ADP[90] and in platelet actin assembly initiated by the fibrinogen receptor, the integrin αIIbβ3[89]. PI 3-kinase plays also a critical role upstream of gelsolin for the exposure of actin filament barbed ends and the actin assembly required for the formation of lamellae mediated by the collagen receptor GPVI[73]. Unpublished data suggest that PI 3-kinase is upstream of Rac in human platelets activated by the other platelet ITAM-containing receptor FcγRIIA (Barkalow, unpublished data).

Conclusion

The platelet cytoskeleton has two main functions: (i) maintain the discoid shape of the resting platelet; and (ii) mediate a rapid shape change in response to external stimuli at sites of vascular injury. At rest, actin-binding and cross-linking proteins such as filamin A and spectrin maintain the unique architecture of the actin cytoskeleton and connect it to the plasma membrane. Capping proteins, such as CapZ and adducin, prevent the assembly of actin onto the barbed ends of the actin filaments composing the resting cytoskeleton. β4–thymosin and profilin maintain a large actin monomer pool that can be rapidly mobilized to assemble into filaments for the shape change reaction following platelet activation. Receptor ligation leads to the cytoplasmic signals, Ca^{2+} and membrane ppIs, which stimulate the activities of the regulatory proteins, gelsolin, adducin and the WASp family proteins. The interactions of these molecules generate actin nucleation sites to initiate actin filament growth by stimulating the Arp2/3 complex to form new barbed ends or by dissociating capping proteins to expose barbed ends. These intimate interactions between cytoplasmic signals and the actin cytoskeleton make the rapid shape change of the platelet possible.

Acknowledgements

Supported by grants from the American Heart Association: 9730137N, NIH: HL56252, HL56949.

Cambridge Books Online © Cambridge University Press, 2009

References

1 Hartwig J, DeSisto M. The cytoskeleton of the resting human blood platelet: Structure of the membrane skeleton and its attachment to actin filaments. *J Cell Biol* 1991; 112: 407–425.

2 Nachmias VT, Yoshida K-I. The cytoskeleton of the blood platelet: a dynamic structure. *Adv Cell Biol* 1988; 2: 181–211.

3 Huxley H. Electron microscopic studies on the structure of natural and synthetic protein filaments from striated muscle. *J Mol Biol* 1963; 3: 281–308.

4 Pollard T. Rate constants for the reactions of ATP- and ADP-actin with the ends of actin filaments. *J Cell Biol* 1986; 103: 2747–2754.

5 Fox JEB, Phillips DR. Inhibition of actin polymerization in blood platelets by cytochalasins. *Nature* 1981; 292: 650–652.

6 Barkalow K, Witke W, Kwiatkowski D, Hartwig J. Coordinated regulation of platelet actin filament barbed ends by gelsolin and capping protein. *J Cell Biol* 1996; 134: 389–399.

7 Nachmias V, Golla R, Casella J, Barron-Casella E. Cap Z, a calcium insensitive capping protein in resting and activated platelets. *FEBS Lett* 1996; 378: 258–262.

8 Kuhlman P, Hughes C, Bennett V, Fowler V. A new function for adducin. Calcium/calmodulin-regulated capping of the barbed ends of actin filaments. *J Biol Chem* 1996; 271: 7986–7991.

9 Li X, Matsuoka Y, Bennett V. Adducin preferentially recruits spectrin to the fast growing ends of actin filaments in a complex requiring the MARCKS-related domain and a newly defined oligomerization domain. *J Biol Chem* 1998; 273: 19329–19338.

10 Carlier M-F, Jean C, Rieger K, Lenfant M, Pantaloni D. Modulation of the interaction between G-actin and thymosin b4 by the ATP/ADP ratio: possible implication in the regulation of actin dynamics. *Proc Natl Acad Sci USA* 1993; 90: 5034–5038.

11 Safer D, Golla R, Nachmias V. Isolation of a 5-kilodalton actin-sequestering peptide from human blood platelets. *Proc Natl Acad Sci USA* 1990; 87: 2536–2539.

12 Carlsson L, Nystrom LE, Sundkvist I, Markey F, Lindberg U. Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells. *J Mol Biol* 1977; 115: 465–483.

13 Markey F, Lindberg U, Eriksson L. Human platelets contain profilin, a potential regulator of actin polymerizability. *FEBS Lett* 1978; 88: 75–79.

14 Ressad F, Didry D, Egile C, Pantaloni D, Carlier M. Control of actin filament length and turnover by actin depolymerizing factor (ADF/cofilin) in the presence of capping proteins and ARP2/3 complex. *J Biol Chem* 1999; 274: 20970–20976.

15 Pavalko F, Otey C, Simon K, Burridge K. Alpha-actinin: a direct link between actin and integrins. *Biochem Soc Trans* 1991; 19: 1065–1069.

16 Ezzell R, Stossel T, Hartwig J. Actin-binding protein (ABP) is associated with a plasma membrane glycoprotein (GPabp) in myeloid and epithelial cell lines. *J Cell Biol* 1989; 109: 191a.

17 Fox J. Identification of actin-binding protein as the protein linking the membrane skeleton to glycoproteins on platelet plasma membrane. *J Biol Chem* 1985; 260: 11970–11977.

18 Andrews R, Fox J. Interaction of purified actin-binding protein with the platelet membrane glycoprotein Ib–IX complex. *J Biol Chem* 1991; 266: 7144–7147.

19 Andrews R, Fox J. Identification of a region in the cytoplasmic domain of the platelet membrane glycoprotein Ib–IX complex that binds to purified actin-binding protein. *J Cell Biol* 1992; 267: 18605–18611.

20 Kovacsovics T, Hartwig J. Thrombin-induced GPIb–IX centralization on the platelet surface requires actin assembly and myosin II activation. *Blood* 1996; 87: 618–629.

21 Bennett V, Gardner K, Steiner JP. Brain adducin: a protein kinase C substrate that may mediate site-directed assembly at the spectrin–actin junction. *J Biol Chem* 1988; 263: 5860–5869.

22 Gardner K, Bennett V. Modulation of spectrin–actin assembly by erythrocyte adducin. *Nature* 1987; 328: 359–362.

23 Bass F, Gaffet P, Bienvenue A. Correlation between inhibition of cytoskeleton proteolysis and anti-vesiculation effect of calpeptin during A23187-induced activation of human platelets: are vesicles shed by filopod fragmentation? *Biochim Biophys Acta* 1994; 1190: 217–224.

24 Fox JEB, Reynolds CC, Phillips DR. Calcium-dependent proteolysis occurs during platelet aggregation. *J Biol Chem* 1983; 258: 9973–9981.

25 White J, Rao G. Microtubule coils versus the surface membrane cytoskeleton in maintenance and restoration of platelet discoid shape. *Am J Path* 1998; 152: 597–609.

26 Kenney D, Linck R. The cytoskeleton of unstimulated blood platelets: structure and composition of the isolated marginal microtubular band. *J Cell Sci* 1985; 78: 1–22.

27 Italiano J, Lecine P, Shivdasani R, Hartwig. Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *J Cell Biol* 1999; 147: 1299–1231.

28 Rothwell S, Calvert V. Activation of human platelets causes post-translational modifications to cytoplasmic dynein. *Thromb Haemost* 1997; 78: 910–918.

29 Tablin F, Castro M. Bovine platelets contain a 280 kDa microtubule-associated protein antigenically related to brain MAP 2. *Eur J Cell Biol* 1991; 56: 415–421.

30 Hartwig J. An ultrastructural approach to understanding the cytoskeleton. In Carraway K, Carraway C, eds. *The cytoskeleton: a practical approach*. Oxford, UK: Oxford University Press; 1992: 23–45.

31 Hartwig J. Mechanism of actin rearrangements mediating platelet activation. *J Cell Biol* 1992; 118: 1421–1442.

32 Yin H, Stull J. Proteins that regulate dynamic actin remodeling in response to membrane signaling; minireview. *J Biol Chem* 1999; 274: 32529–32530.

33 Yin HL, Stossel TP. Control of cytoplasmic actin gel–sol transformation by gelsolin, a calcium-dependent regulatory protein. *Nature* 1979; 281: 583–586.

34 Lind SE, Janmey PA, Chaponnier C, Herbert T-J, Stossel TP.

Cambridge Books Online © Cambridge University Press, 2009

Reversible binding of actin to gelsolin and profilin in human platelet extracts. *J Cell Biol* 1987; 105: 833–842.
35 Janmey P, Stossel T. Modulation of gelsolin function by phosphatidylinositol 4,5-bisphosphate. *Nature* 1987; 325: 362–364.
36 Meerschaert K, Corte VD, Ville YD, Vandekerckhove J, Gettemans J. Gelsolin and functionally similar actin-binding proteins are regulated by lysophosphatidic acid. *EMBO J* 1998; 17: 5923–5932.
37 Schafer D, Jennings P, Cooper J. Dynamics of capping protein and actin assembly in vitro: uncapping barbed ends by polyphosphoinositides. *J Cell Biol* 1996; 135: 169–179.
38 Liu Y, Yin H. Identification of the binding partners for flightless I, a novel protein bridging the leucine-rich repeat and the gelsolin superfamilies. *J Biol Chem* 1998; 273: 7920–7927.
39 Kimura K, Fukata Y, Matsuoka Y et al. Regulation of the association of adducin with actin filaments by Rho-associated kinase (Rho-kinase) and myosin phosphatase. *J Biol Chem* 1998; 273: 5542–5548.
40 Fukata Y, Oshiro N, Kinoshita N et al. Phosphorylation of adducin by Rho-kinase plays a crucial role in cell motility. *J Cell Biol* 1999; 145: 347–361.
41 Joshi R, Bennett V. Mapping the domain structure of human erythrocyte adducin. *J Biol Chem* 1990; 265: 13130–13136.
42 Joshi R, Gilligan D, Otto E, McLaughlin T, Bennett V. Primary structure and domain organization of human alpha and beta adducin. *J Cell Biol* 1991; 115: 665–675.
43 Carlier M, Ducruix A, Pantaloni D. Signalling to actin: the Cdc42-N-WASP-Arp2/3 connection. *Chem Biol* 1999; 6:R235–R240.
44 Higgs H, Pollard T. Regulation of actin polymerization by Arp2/3 complex and WASp/Scar proteins. *J Biol Chem* 1999; 274: 32531–32534.
45 Snapper SB, Rosen FS. The Wiskott–Aldrich syndrome protein (WASP): roles in signaling and cytoskeletal organization. *Annu Rev Immunol* 1999; 17: 905–929.
46 Miki H, Miura K, Takenawa T. N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases. *EMBO J* 1996; 15: 5326–5335.
47 Miki H, Suetsugu S, Takenawa T. WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. *EMBO J* 1998; 17: 6932–6941.
48 Bear JE, Rawls J, CL Saxe. SCAR, a WASP-related protein, isolated as a suppressor of receptor defects in late *Dictyostelium* development. *J Cell Biol* 1998; 142: 1325–1335.
49 Symons M, Derry JM, Karlak B et al. Wiskott–Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization. *Cell* 1996; 84: 723–734.
50 Ramesh N, Anton IM, Martinez-Quiles N, Geha RS. Waltzing with WASP. *Trends Cell Biol* 1999; 9: 15–19.
51 Castellano F, Montcourrier P, Guillemot JC et al. Inducible recruitment of Cdc42 or WASP to a cell-surface receptor triggers actin polymerization and filopodium formation. *Curr Biol* 1999; 9: 351–360.
52 Machesky L, Reeves E, Wientjes F et al. Mammalian actin-related protein 2/3 complex localizes to regions of lamellipodial protrusion and is composed of evolutionarily conserved proteins. *Biochem J* 1997; 328: 105–112.
53 Mullins R, Stafford W, Pollard T. Structure, subunit topology, and actin-binding activity of the Arp2/3 complex from *Acanthamoeba*. *J Cell Biol* 1997; 136: 331–343.
54 Rohatgi R, Ma L, Miki H et al. The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* 1999; 97: 221–231.
55 Welch M, Iwamatsu A, Mitchison T. Actin polymerization is induced by Arp2/3 protein complex at the surface of *Listeria* monocytogenes. *Nature* 1997; 385: 265–269.
56 Welch M, Rosenblatt J, Skoble J, Portnoy D, Mitchison T. Interaction of human Arp2/3 complex and the *Listeria* monocytogenes ActA protein in actin filament nucleation. *Science* 1998; 281: 105–108.
57 Loisel T, Boujemaa R, Pantaloni D, Carlier M. Reconstitution of actin-based motility of *Listeria* and *Shigella* using pure proteins. *Nature* 1999; 401: 613–616.
58 Svitkina T, Borisy G. Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia. *J Cell Biol* 1999; 145: 1009–1026.
59 Oda A, Ochs HD, Druker BJ et al. Collagen induces tyrosine phosphorylation of Wiskott-Aldrich syndrome protein in human platelets. *Blood* 1998; 92: 1852–1858.
60 Gross BS, Wilde JI, Quek L, Chapel H, Nelson DL, Watson SP. Regulation and function of WASp in platelets by the collagen receptor glycoprotein VI. *Blood* 1999; 94: 4166–4176.
61 Rengan R, Ochs HD, Sweet LI et al. Actin cytoskeletal function is spared, but apoptosis is increased, in WAS patient hematopoietic cells. *Blood* 2000; 95: 1283–1292.
62 Egile C, Loisel T, Laurent V et al. Activation of the CDC42 effector N-WASP by the *Shigella flexneri* IcsA protein promotes actin nucleation by Arp2/3 complex and bacterial actin-based motility. *J Cell Biol* 1999; 146: 1319–1332.
63 Hartwig J, Bokoch G, Carpenter C et al. Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. *Cell* 1995; 82: 643–653.
64 Brass LF, Molino M. Protease-activated G protein-coupled receptors on human platelets and endothelial cells. *Thromb Haemost* 1997; 78: 234–241.
65 Offermanns S, Toombs CF, Hu YH, Simon ML. Defective platelet activation in $G\alpha_q$-deficient mice. *Nature* 1997; 389: 183–186.
66 Rasmussen U, Vouret-Craviari V, Jallat S et al. cDNA cloning and expression of a hamster α-thrombin receptor coupled to Ca2+ mobilization. *FEBS Lett* 1991; 288: 123–128.
67 Vu T, Hung D, Wheaton V, Coughlin S. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism for receptor activation. *Cell* 1991; 64: 1057–1068.
68 Watson SP, Gibbins J. Collagen receptor signalling in platelets: extending the role of the ITAM. *Immunol Today* 1998; 19: 260–264.
69 Jandrot-Perrus M, Busfield S, Lagrue A et al. Cloning, charac-

Cambridge Books Online © Cambridge University Press, 2009

terization, and functional studies of human and mouse glycoprotein VI: a platelet-specific collagen receptor from the immunoglobulin superfamily. *Blood* 2000; 96: 1798–1807.

70 Ezumi Y, Shindoh K, Tsuji M, Takayama H. Physical and functional association of the Src family kinases Fyn and Lyn with the collagen receptor glycoprotein VI-Fc receptor γ chain complex on human platelets. *J Exp Med* 1998; 188: 267–276.

71 Clemetson JM, Polgar J, Magnenat E, Wells TNC, Clemetson KJ. The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcaR and the natural killer receptors. *J Biol Chem* 1999; 274: 29019–29024.

72 Clements JL, Lee JR, Gross B et al. Fetal hemorrhage and platelet dysfunction in SLP-76-deficient mice. *J Clin Invest* 1999; 103: 19–25.

73 Falet H, Barkalow KL, Pivniouk VI, Barnes MJ, Geha RS, Hartwig JH. Roles of SLP-76, phosphoinositide 3-kinase and gelsolin in the platelet shape changes initiated by the collagen receptor GPVI/FcRγ-chain complex. *Blood* 2000; 96: 3786–3792.

74 Gross BS, Lee JR, Clements JL et al. Tyrosine phosphorylation of SLP-76 is downstream of Syk following stimulation of the collagen receptor in platelets. *J Biol Chem* 1999; 274: 5963–5971.

75 Poole A, Gibbins JM, Turner M et al. The Fc receptor γ-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *EMBO J* 1997; 16: 2333–2341.

76 Gibbins JM, Briddon S, Shutes A et al. The p85 subunit of phosphatidylinositol 3-kinase associates with the Fc receptor γ-chain and linker for activator of T cells (LAT) in platelets stimulated by collagen and convulxin. *J Biol Chem* 1998; 273: 34437–34443.

77 Pasquet J-M, Bobe R, Gross B et al. A collagen-related peptide regulates phospholipase Cγ2 via phosphatidylinositol 3-kinase in human platelets. *Biochem J* 1999; 342: 171–177.

78 Lagrue AH, Francischetti IM, Guimaraes JA, Jandrot-Perrus M. Phosphatidylinositol 3′-kinase and tyrosine-phosphatase activation positively modulate convulxin-induced platelet activation. Comparison with collagen. *FEBS Lett* 1999; 448: 95–100.

79 Gratacap MP, Payrastre B, Viala C, Mauco G, Plantavid M, Chap H. Phosphatidylinositol 3,4,5-trisphosphate-dependent stimulation of phospholipase C-γ2 is an early key event in FcγRIIA-mediated activation of human platelets. *J Biol Chem* 1998; 273: 24314–24321.

80 Rameh LE, Rhee SG, Spokes K, Kazlauskas A, Cantley LC, Cantley LG. Phosphoinositide 3-kinase regulates phospholipase Cγ-mediated calcium signaling. *J Biol Chem* 1998; 273: 23750–23757.

81 Saci A, Pain S, Rendu F, Bachelot-Loza C. Fc receptor-mediated platelet activation is dependent on phosphatidylinositol 3-kinase activation and involves p120^{cbl}. *J Biol Chem* 1999; 274: 1898–1904.

82 Nobes CD, Hall A. Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 1995; 81: 53–62.

83 Tolias K, Hartwig J, Ishihara H, Shibasaki Y, Cantley L, Carpenter C. Type I-alpha phosphatidylinositol-4-phosphate 5-kinase mediates Rac-dependent actin assembly. *Curr Biol* 2000; 10: 153–156.

84 Azim AC, Barkalow KL, Chou J, Hartwig JH. Activation of the small GTPases, rac and cdc42, after ligation of the platelet PAR-1 receptor. *Blood* 2000; 95: 959–964.

85 Han J, Luby-Phelps K, Das B et al. Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. *Science* 1998; 279: 558–560.

86 Gringhuis SI, de-Leij LF, Coffer PJ, Vellenga E. Signaling through CD5 activates a pathway involving phosphatidylinositol 3-kinase, Vav, and Rac1 in human mature T lymphocytes. *Mol Cell Biol* 1998; 18: 1725–1735.

87 Miranti CK, Leng L, Maschberger P, Brugge JS, Shattil SJ. Identification of a novel integrin signaling pathway involving the kinase Syk and the guanine nucleotide exchange factor Vav1. *Curr Biol* 1998; 8: 1289–1299.

88 Missy K, van-Poucke V, Raynal P et al. Lipid products of phosphoinositide 3-kinase interact with Rac1 GTPase and stimulate GDP dissociation. *J Biol Chem* 1998; 273: 30279–30286.

89 Hartwig J, Kung S, Kovacsovics T et al. D3 phosphoinositides and outside-in integrin signaling by GPIIb/IIIa mediate platelet actin assembly and filopodial extension induced by phorbol 12-myristate 13-acetate. *J Biol Chem* 1996; 271: 32986–32993.

90 Heraud J-M, Racaud-Sultan C, Gironcel D et al. Lipid products of phosphoinositide 3-kinase and phosphatidylinositol 4′,5′-bisphosphate are both required for ADP-dependent platelet spreading. *J Biol Chem* 1998; 273: 17817–17823.

Cambridge Books Online © Cambridge University Press, 2009

7

Platelet organelles

Francine Rendu and Brigitte Brohard-Bohn

Centre de médecine préventive cardiovasculaire, Hôpital Broussais, Paris, France

Introduction

The blood platelet is unique in that it appears as a very simple cell with a clear cytoplasm, no nucleus, and a variety of organelles. These organelles contain various components which serve in metabolic and secretory processes. In response to a variety of stimuli, platelets liberate granule constituents in the outer medium. Yet, platelets cannot be considered a secretory cell, since they do not synthesize the secreted components. The purpose of this chapter is to describe the different platelet organelles, to analyse the role of each of them and to describe the function of the different granule constituents.

The different organelles, including few mitochondria, are dispersed randomly in the cytoplasm together with skeletal components (microtubules and actin filaments), a large amount of glycogen as a source of energy, and a complex membranous system. The latter consists of two prominent structures: the open canalicular system, which allows connections between the cytosol and the surrounding medium, and the dense tubular system (DTS), which stores important metabolic enzymes. Mitochondria and DTS are involved in metabolic processes, whereas specific granules (dense granules, alpha granules, and lysosomes) are involved in platelet secretion, the so-called 'platelet release reaction'. In other words, mitochondria and DTS work in concert to provide the metabolic energy and control the cytosolic calcium required for secretion of the different granule constituents.

Dense granules (also termed dense bodies, dense core granules, and δ-granules)

These are so-called because they are both heavy and electron dense.

Structure, size, and number

Dense granules are the smallest granules present in the platelet cytoplasm, with an average diameter of 150 nm. Typically, dense granules are characterized by the presence of an intensely opaque dense core surrounded by a clear space and a single membrane. The dense body may completely fill the whole lumen of the granule, or depending on the thin section of the electron microscope grid, it appears round, spherical, elongated or even with a round head and a tail as a tadpole. There is an average of 5–6 dense granules per platelet[1–3].

Properties

Dense granules have three major characteristics: (i) they absorb the electron beam, and hence are intrinsically dense when viewed by electron microscopy of unstained whole mount platelet preparations. Inasmuch, being highly osmophilic, they appear black after osmium staining of thin sections; (ii) they contain small non-protein molecules; (iii) they are able to accumulate various basic compounds, such as reserpine, chlorpromazine, chloroquine and mepacrine[4]. Dense granules also incorporate serotonin through a very high concentration gradient, and the amine concentration within the dense granule can be a thousand times higher than in plasma. The latter property is related to the fact that the internal pH of the dense granule is acidic (pH = 6.1), a pH maintained thanks to a proton-pumping ATPase[5]. Thus, dense granule properties are very similar to those of adrenal chromaffin granules, storing catecholamines.

Content

Compounds stored in dense granule are small molecules (Table 7.1). Dense granules contain high concentrations of

Table 7.1. Platelet dense granule constituents

Membrane	Matrix
GTP binding protein: Ral1	Adenine nucleotides: ATP, ADP
Glycoproteins GPIb[a,b], IIbIIIa[a,b]	Serotonin (5-HT)
Granulophysin (LIMP1/CD63)[c]	Calcium, magnesium
LAMP 2[c]	Pyrophosphate
Src[b]	Guanine nucleotides: GTP, GDP
P-selectin (CD62)[a,b]	Histamine

Notes:
[a] Also present on alpha-granule membrane.
[b] Also present on plasma membrane.
[c] Also present on lysosomal membrane.

adenine nucleotides (65% of total platelet content) with over 0.6 and 0.4 M of ADP and ATP, respectively. This pool of adenine nucleotides is metabolically inert and can be distinguished from the metabolic pool in that it is not labelled by incubation of platelets with radiolabelled phosphate. They also contain 70% of the total platelet content of bivalent cations, with calcium as the predominant ion in human platelets at around 2.2 M (Table 7.1). This pool of calcium is distinct from the pool which is mobilized for platelet activation, the latter being localized in the dense tubular system. Dense granules are the storage granules for serotonin (5-hydroxytryptamine), at a concentration of 65 mM, and hence were originally called the 5-hydroxytryptamine organelles. Serotonin is the major osmiophil factor of dense granules. Within the dense core, adenine nucleotides and pyrophosphate form tight complexes with calcium and serotonin held together by intermolecular forces[6]. Consequently, the high amount of calcium, not in an ionised form but in a heavy complex, confers to denser granules their high stability. A high level of phosphorus also participates in the high dense granule stability[7].

Membrane

As for classical exocytotic vesicles, the dense granule membrane consists of phospholipids with insertion of proteins[8]. Analysis of phospholipids revealed that dense granules are rich in lysolecithin and ganglioside GM3, a property shared with chromaffin granules[9]. A small GTP-binding protein, Ral, is associated with the dense granule membrane[10], suggesting that the dense granule is equipped of transducing proteins required for the release reaction (Table 7.1). After the secretion process, the granule membrane is integrated in the platelet membrane and yields the expression of dense granule membrane proteins on the activated platelet surface. This is the case for granulophysin[11], and the tyrosine kinase src[12,13]. Noteworthy, a number of receptors which are present on either plasma membrane or other granule membrane, are also present on the dense-granule membrane. Granulophysin is identical to LIMP-1, a lysosomal integral membrane protein (see lysosome membrane section)[11]. The glycoproteins GPIb and GPIIbIIIa[14] and P-selectin[15] are also present on plasma and alpha-granule membranes.

Origin

Dense granules are formed in the megakaryocyte (MK) at an early stage of maturation[16]. In MK, they are empty sacks which are filled in with adenine nucleotides during maturation[17], and with serotonin at an intermediate stage. In mature MK, dense granules are less numerous per unit of surface than in platelets [18].

Means of study

The osmiophilic property of dense granules together with their intrinsic density allows their clear distinction from other granules by electron microscopy, either by whole mount technique[3], or by the uranaffin reaction, specific for phosphonucleotides[19]. Dense granule capacity to incorporate serotonin has been widely used to study their release during platelet activation, after preincubation of platelets with radiolabelled serotonin which is incorporated almost exclusively in the dense granule. The high density and stability of dense granules has allowed their isolation by subcellular fractionations on various types of gradients at high yields and purity[9,20,21], inasmuch as their purification could be followed by direct observation of mepacrine-labelled granules. Mepacrine has also allowed their quantitation by direct observation under a fluorescence microscope[1,2], and is now widely used in flow cytometry to monitor the yellow-green fluorescence of platelet dense granule content[22]. It can also be used to monitor the dense granule release[23] and advantageously replace the radiolabelling of dense granule serotonin.

Functions

Dense granules contain high levels of ADP and calcium, their function during the release reaction is therefore to liberate in the outer medium ‘proaggregating factors’ that will recruit other platelets to aggregate. Serotonin is also a proaggregating agent although this does not hold true in every species. In human, serotonin is a weak platelet agonist, and it most likely acts as a local vasoconstrictor at

Cambridge Books Online © Cambridge University Press, 2009

the site of injury which will in turn contribute to further aggregate platelets.

Defects in dense granules

Isolated dense granule deficiency remains rare, although its incidence may be high in some populations, as is the case for the Hermansky–Pudlak syndrome (see below) frequent in northwestern Puerto Rico. Dense granule deficiency is referred to as δ-storage pool deficiency (SPD) identifying the defective granule (δ for dense granule). Criteria for this disease include reduced dense granule number, reduced platelet content of dense granule constituents (decreased ADP and decreased serotonin), and hence defective dense granule functions[24,25]. The dense granule structure abnormality is very heterogeneous[26,27]. The clinical manifestation is characterized by a moderate bleeding from bruising and epistaxis to bleeding after surgery. The laboratory screening reveals a prolonged bleeding time and a typical platelet aggregation defect, characterized by the absence of a second wave of aggregation or rapid deaggregation in response to ADP, and no aggregation in response to low doses of collagen.

δ-SPD is often associated with pigment abnormalities. Actually, the first δ-SPD was described by Hermansky and Pudlak[28], who reported two cases presenting with hemorrhagic disorder of medium severity with a prolonged bleeding time concomitant with tyrosine-positive oculocutaneous albinism and accumulation of an unusual ceroidlike pigment in macrophages. The Hermansky–Pudlak syndrome (HPS) may be complicated by different diseases, including granulomatous colitis and inflammatory and lung diseases. The latter may be directly related to the platelet dense granule defect: because the defective platelet does not store serotonin properly, the amine is at a higher level in the plasma. In a case of HPS associated with pulmonary hypertension, administration of ketanserine, an antagonist of serotonin, reduced the pulmonary hypertension[29]. The Chediak–Higashi syndrome (CHS)[30] also falls in this category of platelet δ-SPD[31] and is associated with deficient pigmentation, the presence of large granules in leukocytes and immune deficiency. The two syndromes, HPS and CHS have been recently reviewed[32]. There is no specific treatment for δ-SPD. The use of desmopressin (1-deamino-8–D-arginine vasopressin, DDAVP) may be helpful, however, to prevent excess bleeding and to shorten the bleeding time.

The genes for each of the two syndromes HPS and CHS have recently been identified with a number of mutations identified. The HPS gene encodes a 79 kD HPS protein and the most important pathologic frameshift mutations of the gene abolish formation of functional HPS protein, suggesting that truncation of HPS proteins may have consequences at the subcellular level[33]. As with HPS, the CHS gene encodes a CHS protein, and frameshift and nonsense mutations abolish expression of CHS protein[33]. In the two syndromes, however, the above mutations were not found in every patient, and other loci in the genome and/or other factors may determine the clinical phenotype.

Alpha granules

This population of granules is much more heterogeneous than the previous one, both in terms of opacity and structure and of constituents. They represent the major granule population in size and in number[35].

Structure, size and number

Alpha granules are large organelles of 200–400 nm diameter with a single membrane. They are spherical or ovoid granules, with an electron density lower than that of dense granules and not even in the granule lumen[36]. Two major compartments can be identified: the dark nucleoid containing proteoglycans, and an electron-luscent matrix[37]. The latter can be subdivided into the nucleoid adjacent region, an intermediate zone often associated with labelling with plasmatic proteins, and a small peripheral zone characterized by the presence of tubular structures where the massive proteins, von Willebrand Factor (vWF), GPIa/multimerin, and factor V are colocalized[38,39].

Properties

The alpha-granule population has two major characteristics: (i) the complex mechanism of packaging of the protein content by both synthesis and endocytosis; and (ii) the nature and functions of the proteins stored, which are large adhesive and healing proteins. The alpha granules are typical secretory vesicles which carry proteins to the cell surface to be released: some of the released soluble intragranular proteins adhere to the platelet surface and become peripheral proteins of the plasma membrane; some are incorporated in the membrane; and some diffuse into the extracellular fluid. Thus, alpha granules concentrate plasma proteins that play a critical role in hemostasis, wound healing and cell–matrix interactions, and store them until delivery is required at a site of vessel injury.

Cambridge Books Online © Cambridge University Press, 2009

Table 7.2. Platelet alpha-granule constituents

Membrane	Matrix
GTP binding proteins	*Proteoglycans*
rab4, GMP33, Rap1[b]	platelet specific: βTG[c], PF4[c]
Receptors and antigens	serglycin, HRGP
P-selectin[a] (CD62)	βTG Ag molecules: PBP, CTAP-III, NAP-2
GPIIbIIIa[a,b]	*Adhesive glycoproteins*
GPIb–IX[b]	Fibronectin[d], vitronectin, vWF[c], thrombospondin[c]
GPIV (CD36)[b]	*Haemostasis factors and cofactors*
p24 (CD9)[b]	Fibrinogen[d], Factor V[c], VIII, XI, XIII
PECAM (CD 31)[b]	Kininogens, protein S, plasminogen
GLUT-3	*Cellular mitogens*
Vitronectin receptor	PDGF, TGFβ, ECGF, EGF, VEGF/VPF, IGF,
	Interleukin β
	Protease inhibitors
Osteonectin	α_2-macroglobulin, α_2-antitrypsin, PDCI
	α_2-antiplasmin, PAI1, TFPI
	PN-2/APP, C1 inhibitor
	Miscellaneous
	Immunoglobulins[d]: IgG, IgA, IgM
	Albumin[d], GPIa/multimerin

Notes:
[a] Also present on dense-granule membrane.
[b] Also present on plasma membrane.
[c] Synthesized in the megakaryocytes.
[d] Endocytosed from plasma.
See text for abbreviations.

Content

Two specific platelet proteins, i.e. β-thromboglobulin (βTG) and platelet factor 4 (PF4), are localized in the alpha-granule nucleoid together with proteoglycans (Table 7.2). The latter include a chondroitin sulfate, serglycine, an histidine rich glycoprotein (HRGP), and a family of βTG-antigen (Ag) molecules consisting of the platelet basic protein (PBP), connective tissue activating protein-III (CTAP-III) and neutrophil-activating protein-2 (NAP-2), all precursors of βTG and PF4. Proteoglycans confer to the alpha-granules a relative stability. Besides these proteoglycans, alpha-granules contain many different types of large proteins, such as adhesive proteins, coagulation factors, and cellular mitogens. Among the mitogenic factors, the specific platelet derived growth factor (PDGF) is present together with transforming growth factor β (TGFβ), epidermic growth factor (EGF), endothelial cell growth factor (ECGF), transforming growth factor-β (TGF-β), insulin-like growth factor (IGF), vascular permeability factor (VPF) or vascular endothelial growth factor (VEGF), and interleukin β. Protease inhibitors are also present in the alpha granules, among which are the platelet derived collagenase inhibitor (PDCI)[40], the protease nexin-2/amyloid beta-protein precursor (PN-2/APP), and the plasminogen activator inhibitor 1 (PAI 1) tissue factor plasminogen inhibitor (TFPI). The coagulation factors and some other proteins, such as albumin, are identical or similar to the corresponding plasma proteins. Fibrinogen, thrombospondin and fibronectin are detected only in the intermediate zone of the granule lumen.

Membrane

The major alpha-granule membrane protein was simultaneously discovered in two different groups: the platelet activated derived granule external membrane (PADGEM)[41] or the granule membrane protein 140 (GMP140)[42], is now termed P-selectin, and is the CD62 antigen. Other glycoproteins are found at the inner face of the alpha-granule membrane, including GPIIbIIIa, GPIb–IX, GPIV (CD36), the tetraspanin CD9,[43] GMP-33,[44] and osteonectin[45]. The

Cambridge Books Online © Cambridge University Press, 2009

glucose transporter GLUT-3[46], the platelet endothelial cell adhesion molecule (PECAM) and the vitronectin receptor[47] are also present on the alpha-granule membrane. As for the dense granule, the alpha-granule membrane is equipped with GTP-binding proteins (Rap1, rab4, rab6 and rab8) required for the regulatory mechanism of secretion, although the mechanisms differ for alpha and dense granule populations[48–50].

Origin

Alpha granules are formed during MK maturation. In immature MKs, small immature alpha granules are found which enlarge during MK maturation. The gradual morphological transition occurs through different types of multivesicular bodies[51]. Some of the alpha-granule proteins are synthesized by the MK (βTG, PF4, vWF, factor V), and are expressed early during MK maturation. The proteins synthesized in the MK rough-endoplasmic reticulum are sorted in the *trans*Golgi network, where they are packaged in storage vesicles. The latter pinch off from the network and are visualized in the MK. Other proteins are endocytosed from the circulating plasma pool at both MK and platelet levels, and appear later in MK maturation than synthesized proteins. Endocytosis of proteins that are found at very low concentrations in the alpha-granule, such as immunoglobulins and albumin, proceeds passively by the so-called fluid phase endocytosis. Endocytosis of proteins that are present at a concentration within the alpha granule higher than in plasma, occurs through a receptor-mediated endocytotic process. This is the case for fibrinogen that is endocytosed through the GPIIbIIIa receptor.

Means of study

The two platelet specific proteins βTG and PF4 can be monitored by immunoassays. Hence, they are useful tools to monitor both the amount and the release of alpha granules, using commercially available immunoassays. The expression of P-selectin on the activated platelet membrane is now widely used as a marker of platelet activation and is easily detected by flow cytometry using an anti P-selectin antibody.

Functions

The function of alpha-granules during platelet activation is to release ‘repairing factors’ in the outer medium in the context of wounding. Newly expressed GPIIbIIIa, and its bound ligands, fibrinogen and vWF, contribute to magnify aggregation and above all to the spreading of aggregated platelets on exposed subendothelium. In specific activation conditions, secreted platelet factor V binds to the activated platelet surface possibly on the exposed complex GPIa/multimerin, and hence serves as a receptor for factor Xa, thus favouring coagulation[52]. Thrombospondin and osteonectin, members of the secreted protein, acidic and rich in cystein (SPARC) family, form multimolecular complexes with other adhesive proteins (albumin and PDGF among others) on the extracellular matrix of the platelet surface and mediate interactions with basement membranes involved in tissue remodelling at sites of wound repair[53,54]. The P-selectin exposed on the activated platelet membrane binds its counter receptor, the P-selectin glycoprotein ligand 1 (PSGL1) present on leukocytes and endothelial cells and is thus responsible for platelet–leukocyte and platelet–endothelial cell interactions[55,56]. The βTG-Ag molecules are secreted in unactive forms of precursors of chemokines ready to control the inflammation process[57]. The two platelet proteins, PF4 and βTG, and HRGP have antiheparin activity, and would modulate FGF activity by competing with the latter on cell surface heparan sulfate proteoglycans, making FGF available[58]. Serglycin binds to PF4 and collagen and regulates the inflammatory process, inasmuch as it binds the lymphocyte adhesion molecule CD44[59]. The PN-2/ APP inhibits blood coagulation, a function of special importance in the brain, since the APP processing is altered in platelets of patients with Alzheimer disease[60]. Mitogenic factors have various roles in wound repair once liberated. For example, PDGF stimulates medial smooth muscle cell proliferation, whereas TGFβ inhibits cell proliferation; both PDGF and TGFβ stimulate collagen synthesis.

Defects in alpha granules

The referenced alpha-granule deficiency is the Gray syndrome, so called because the lack of alpha granules confers to the platelet a grey colour after May–Grunwald–Giemsa staining of a platelet smear. It is a rare congenital disorder, first described by Raccuglia[61], and characterized by a bleeding tendency associated with quantitative and qualitative platelet abnormalities. Intensity of the hemorrhagic syndrome is moderate and varies with the thrombopenia. The mean platelet volume is higher than normal, and platelet size is heterogeneous. Platelet functions are decreased in most reported cases[62–64]. The platelet life span is slightly shorter than normal despite a normal number of MK. The granule membrane is normal, hence platelets from patients with Gray syndrome exhibit many vacuoles corresponding to empty alpha granules. Typically, soluble

Cambridge Books Online © Cambridge University Press, 2009

proteins synthesized in the MK are absent, whereas endocytosed proteins are only partially reduced. This is due to a defect of packaging at the MK level where synthesized proteins are not properly targeted to their granule[65]. One characteristic of this syndrome is the frequent observation of myelofibrosis, a consequence of the protein packaging defect: if growth factors and PF4 are normally synthesized but not packaged, they are not properly targeted and are discharged in the extracellular medium through MK demarcation membranes. The disease is thus a model to investigate the protein sorting and packaging in secretory cells.

Another related disease has been described, the factor V Quebec syndrome. This syndrome is characterized by multiple abnormalities concerning alpha-granule proteins, including a quantitative deficiency in mutimerin, and qualitative defects of factor V, thrombospondin, vWF, and fibrinogen[66].

Lysosomes

The third category of granules involved in the release reaction concerns lysosomes or the λ-granules. Lysosomes are lighter than alpha granules and contain a variety of digestive enzymes active at acid pH.

Structure

The size of lysosomes is intermediate between dense granules and alpha granules, measuring 175–250 nm in diameter. They can be identified only by specific cytochemical stains as acid phosphatase or arylsulfatase reactive granules[67]. Without cytochemical stains, their electron density is very similar to that of alpha granules, although this density fills almost the entire granule. No debris has ever been identified inside platelet lysosomes, suggesting they are primary lysosomes.

Properties

The lysosome characteristic is that they contain enzymes which hydrolyse the glucidic moiety of glycoproteins, glycolipids and glycosaminoglycans, i.e. all components of extracellular matrix.

Content

These acidic granules contain almost exclusively acid hydrolases: glycosidases, acid proteases, cationic proteins with bactericidal activity (Table 7.3) which breakdown their substrates to the simplest units, i.e. amino acids, sugars and simple lipids from glycoproteins and glycolipids. The major ones, i.e. which are in sufficient amounts to be used as markers, are β-*N*-acetylglucosaminidase, β-*N*-acetylgalactosaminidase, β-glucuronidase, and β-galactosidase. Others are only present in small amounts.

Table 7.3. Platelet lysosomal constituents

Membrane	Matrix
LIMP1/CD63[a]	*Acid Proteases*
	Cathepsines D, E
LAMP1, LAMP2	Carboxypeptidases (A, B)
	Prolinecarboxypeptidase
	Collagenase
	Acid phosphatase
	Arylsulfatase
	Glycohydrolases
	Heparinase
	β-*N*-acetyl-glucosaminidase,
	β-glucuronidase, β-galactosidase,
	β-glycerophosphatase, α-D-glucosidase,
	β-D-glucosidase, α-L-fucosidase,
	β-D-fucosidase, α-L-arabinosidase,
	α-D-mannosidase,

Note:
[a] Also present on dense granule membrane (see Table 7.1)

Membrane

The lysosome membrane contains typical lysosomal associated membrane proteins (LAMPs), the first described as LIMP/CD63 and two distinct LAMP-1 and LAMP-2[68]. All are heavily glycosylated at the luminal side of the lysosome, probably representing a protective coat against the lysosomal hydrolases. The LIMP/CD63 antigen belongs to the tetraspanin family, which consists of proteins with four transmembrane domains and the two N- and C- extremities in the cytoplasm. Both LIMP/CD 63 and LAMP- 2 are also found on dense granule membrane[11,69].

Origin

Lysosomes are found in the earliest recognizable megakaryocytes. Two lysosomal enzymes can be found in immature MK in small vesicles similar to Golgi-endoplasmic reticulum lysosomes of around 70 nm. These vesicles fuse to form larger ones during MK maturation and increase in number[67].

Cambridge Books Online © Cambridge University Press, 2009

Means of study

All proteases can be monitored spectrophotometrically, with different substrates[70]. The expression of CD63 on the activated platelet surface is now monitored by flow cytometry, and as for alpha-granule P-selectin, used as a marker of platelet activation.

Functions

The typical lysosomal role of desensitisation of internalised receptors, such as described for the thrombin receptor[71], seems limited in this anucleated cell unable to resynthesise new receptors. On the other hand in activated platelets, platelet lysosomes play an important role during the release reaction, since they liberate 'clearing factors' in the outer medium. Secretion of acid hydrolases from lysosomes promotes clearing of platelet aggregates and digestion of material trapped in the platelet aggregate. Thus, lysosomes could facilitate the digestion and resolution of thrombi. Noteworthy, lysosome secretion is (i) slower than dense granule and alpha-granule secretion, and (ii) always incomplete even with high agonist concentrations. Yet, upon activation, platelets release their lysosomal content in vivo[72]. It is not unlikely, therefore, that in some pathological conditions, platelets represent an important source of hydrolytic enzymes which play a role in local degradation of the connective tissue matrix of the vascular wall leading to atherosclerosis and focal thickening of arteries.

Defects in lysosomes

So far, no defects specifically concerning platelet lysosomes have been described. Moreover, considering dense granules as a subpopulation of lysosomes[73], it is most likely that any defect of this category will concern dense granules rather than lysosomes.

Conclusions

From this review, it appears that the best characterized platelet granules are the dense and alpha granules. The typical density and properties of dense granules have allowed their easy distinction from other granules. The complexity of the alpha granule has emphasized their major role in hemostasis. For several years, serotonin has been used as the only marker for the release reaction: platelets were incubated with radiolabelled serotonin which was rapidly and exclusively concentrated in the dense granules, and its liberation outside the activated platelet was easily measured. For a more complete screening of the three granule populations, PF4 and βTG, on the one hand, and acid phosphatases, on the other hand, have more rarely been used to measure content and release of alpha granules and lysosomes, respectively. Nowadays, flow cytometry has been substituted as a measure of secreted compounds in the external milieu and is used to analyse activated membranes which have incorporated granule membrane markers. Flow cytometry does not allow, however, the measure of the intact platelet granules, except for the mepacrine-labelled dense granules. Finally, although dense granules are classified in the category of lysosome-related organelles[73], and the different granule membranes share common molecules, the three types of granules have different roles to play during hemostasis and secrete different amount of constituents depending on the conditions of platelet activation.

References

1 Lorez HP, Da Prada, M, Rendu F, Pletscher A. Mepacrine, a tool for investigating the 5-hydroxytryptamine organelles of blood platelets by fluorescence microscopy. *J Lab Clin Med* 1977; 89, 200–206.

2 Skaer RJ, Flemans RJ, McQuilkan S. Mepacrine stains the dense bodies of human platelets and not platelet lysosomes. *Br J Haematol* 1981; 49: 435–438.

3 White JG. Use of electron microscope for diagnosis of platelet disorders. *Sem Thromb Haemost* 1998; 24: 163–168.

4 DaPrada M, Pletscher A. Storage of exogenous monoamines and reserpine in 5-hydroxytryptamine organelles of blood platelets. *Eur J Pharmacol* 1969; 34: 591–597.

5 Dean GE, Fishkes H, Nelson PJ, Rudnick G. The hydrogen ion-pumping adenosine triphosphatase of platelet dense granule membrane. Differences from F1F0– and phosphoenzyme-type ATPases. *J Biol Chem* 1984; 259: 9569–9574.

6 Pletscher A, Da Prada M, Berneis KH, Tranzer JP. New aspects on the storage of 5-hydroxytryptamine in blood platelets. *Experientia* 1971; 27: 993–1120.

7 Skaer RJ, Peters PD, Emmines JP. The localization of calcium and phosphorus in human platelets. *J Cell Sci* 1974; 15: 679–692.

8 DaPrada M, Pletscher A, Tranzer JP. Lipid composition of membranes of amine storage organelles. *Biochem J* 1972; 127: 681–683.

9 Chatterjee D, Anderson GM. The human platelet dense granule: serotonin uptake, tetrabenazine binding, phospholipid and ganglisode profiles. *Arch Biochem Biophys* 1993; 302: 439–446.

10 Mark BL, Jilkina O, Bhullar RP. Association of Ral1A and Ral1B GTP-binding proteins and determination of their concentration and distribution in human platelets. *Biochim Biophys Acta* 1996; 1314: 157–166.

Cambridge Books Online © Cambridge University Press, 2009

11 Nishibori M, Cham B, McNicol A, Shalev A, Jain N, Gerrard JM. The protein CD63 is in platelet dense granules, is deficient in a patient with Hermansky–Pudlak syndrome, and appears identical to granulophysin. *J Clin Invest* 1993; 91: 1775–1782.
12 Rendu F, Lebret M, Danielian S, Fagard R, Levy-Toledano S, Fischer S. High pp60c-src level in human platelet dense bodies. *Blood* 1989; 73: 1545–1551
13 Rendu F, Martin-Cramer E, Tenza D, Fischer S. Redistribution of granulophysin and SRC protein in normal and Gray platelets after activation. *Platelets* 1993; 4: 167–174.
14 Youssefian T, Masse JM, Rendu F, Guichard J, Cramer EM. Platelet and megakaryocyte dense granule contain glycoproteins Ib and IIb–IIIa. *Blood* 1997; 89: 4047–4057.
15 Israels SJ, Gerrard JM, Jacques YV et al. Platelet dense granule membranes contain both granulophysin and P-selectin GMP-140. *Blood* 1992; 80: 143–152.
16 Youssefian T, Cramer EM. Megakaryocyte dense granule components are sorted in multivesicular bodies. *Blood* 2000; 95: 4004–4007.
17 Tranzer JP, Da Prada M, Pletscher A. Storage of 5-hydroxytryptamine in megakaryocytes. *J Cell Biol* 1972; 52: 191–197.
18 Hourdille P, Fialon P, Belloc F, Boisseau MR, Andrieu JM. Mepacrine labelling test and uranaffin cytochemical reaction in human megakaryocytes. *Thromb Haemost* 1982; 47: 232–235.
19 Richards JG, Da Prada M. Uranaffin reaction: a new cytochemical technique for the localization of adenine nucleotides in organelles storing biogenic amines. *J Histochem Cytochem* 1977; 25: 1322–1326.
20 Fukami MH, Bauer JS, Stewart GJ, Salganicoff L. An improved method for the isolation of dense storage granules from human platelets. *J Cell Biol* 1979; 77: 389–399.
21 Rendu F, Lebret M, Nurden AT, Caen JP. Initial characterization of human platelet mepacrine-labelled granules isolated using a short metrizamide gradient. *Br J Haematol* 1982; 52: 241–251.
22 Gordon N, Thom J, Cole C, Baker R. Rapid detection of hereditary and acquired platelet storage pool deficiency by flow cytometry. *Br J Haematol* 1995; 89: 117–123.
23 Wall JE, Buijs-Wilts M, Arnold JT et al. A flow cytometric assay using mepacine for study of uptake and release of platelet dense granule contents. *Br J Haematol* 1995; 89: 380–385.
24 Rendu F, Dupuy E. Hereditary disorders of platelet function. In: Harris JR, ed. *Blood cell biochemistry*, Vol 2. New York: Plenum Publishing Corporation; 1991: 77–112.
25 McNicol A, Israels SJ. Platelet dense granules: structure, function and implications for haemostasis. *Thromb Res* 1999; 95: 1–18.
26 Rendu F, Breton-Gorius J, Trugnan G et al. Studies on a new variant of the Hermansky-Pudlak syndrome: qualitative ultrastructural and functional abnormalities of the platelet dense bodies associated with a phospholipase A defect. *Am J Hematol* 1978; 4: 387–399.
27 Weiss HJ, Lages B, Vicic W, Tsung LY, White JG. Heterogeneous abnormalities of platelet dense granule ultrastructure in 20 patients with congenital storage pool deficiency. *Br J Haematol* 1993; 83: 282–295.
28 Hermansky F, Pudlak P. Albinism associated with hemorrhagic diathesis and unusual pigmented reticular cells in the bone marrow: report of two cases with histochemical studies. *Blood* 1959; 14: 162–169.
29 Herve P, Drouet L, Dosquet C et al. Primary pulmonary hypertension in a patient with a familial platelet storage pool disease: role of serotonin. *Am J Med* 1990; 89: 117–120.
30 Dent PB, Fish LA, White JC, Good RA. Chediak-Higashi syndrome: observation on the nature of the associated malignancy. *Lab Invest* 1966; 15: 1634–1642.
31 Rendu F, Breton-Gorius J, Lebret M et al. Evidence that abnormal platelet functions in human Chediak–Higashi syndrome are the result of a lack of dense bodies. *Am J Pathol* 1983; 111: 307–314.
32 Spritz RA. Molecular genetics of the Hermansky-Pudlak and Chediak–Higashi syndromes. *Platelets* 1998; 9: 21–29.
33 Oh J, Bailin T, Fukai K et al. Positional cloning of a gene for Hermansky–Pudlak syndrome, a disorder of cytoplasmic organelles. *Nature Gen* 1996; 14: 300–306.
34 Spritz RA. Hermansky–Pudlak syndrome and pale ear: melanosome-making for the millennium. *Pigment Cell Res* 2000; 13: 15–20.
35 Harrison P, Cramer EM. Platelet α-granules. *Blood Rev* 1993; 7: 52–62.
36 Harrison P, Savidge GF, Cramer EM. The origin and physiological relevance of alpha-granule adhesive proteins. *Br J Haematol* 1990; 74: 125–130.
37 Suzuki H, Katagiri Y, Tsukita S, Tanoue K, Yamazaki H. Localization of adhesive proteins in two newly subdivided zones in electron-lucent matrix of human platelet α-granules. *Histochem* 1990; 94: 337–344.
38 Hayward CPM, Furmaniak-Kazmierczak E, Cieutat A-M et al. Factor V is complexed with multimerin in resting platelet lysates and colocalises with multimerin within alpha-granules. *J Biol Chem* 1995; 270: 19217– 19224.
39 Polgar J, Magnenat E, Wells TN, Clemetson KJ. Platelet glycoprotein Ia* is the processed form of multimerin. Isolation and determination of N-terminal sequences of stored and released forms. *Thromb Haemost* 1998; 80: 645–648.
40 Copper TW, Eisen AZ, Stricklin GP, Welgus HG. Platelet-derived collagenase inhibitor: characterization and subcellular localization. *Proc Natl Acad Sci USA* 1985; 82: 2779–83.
41 Larsen E, Celi A, Gilbert GE, Furie BC et al. PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. *Cell* 1989; 59: 305–312.
42 Johnston GI, Cook RG, McEver RP. Cloning of GMP140, a granule membrane protein of platelets and endothelium; sequence similarity to proteins involved in cell adhesion and inflammation. *Cell* 1989; 56: 1033–1044.
43 Brisson C, Azorsa DO, Jennings LK, Moog S, Cazenave JP, Lanza F. Co-localization of CD9 and GPIIb–IIIa (alpha IIb beta 3 integrin) on activated platelet pseudopods and alpha-granule membranes. *J Histochem* 1997; 29: 153–165.

Cambridge Books Online © Cambridge University Press, 2009

44 Metzelaar MJ, Heijnen HF, Sixma JJ, Nieuwenhuis HK. Identification of a 33–kD protein associated with the alpha-granule membrane (GMP-33) that is expressed on the surface of activated platelets. *Blood* 1992; 79: 372–379.
45 Breton-Gorius J, Clezardin P, Guichard J et al. Localization of platelet osteonectin at the internal face of the alpha-granule membranes in platelets and megakaryocytes. *Blood* 1992; 79: 936–941.
46 Heijnen HF, Oorschot V, Sixma JJ, Slot JW, James DE. Thrombin stimulates glucose transport in human platelets via the translocation of the glucose transporter GLUT-3 from alpha-granules to the cell surface. *J Cell Biol* 1997; 138: 323–330.
47 Poujol C, Nurden AT, Nurden P. Ultrastructural analysis of the distribution of the vitronectin receptor (alpha v beta 3) in human platelets and megakaryocytes reveals an intracellular pool and labelling of the alpha-granule membrane. *Br J Haematol* 1997; 96: 823–835.
48 Berger G, Quarck R, Tenza D, Levy-Toledano S, de Gunzbourg J, Crame EM. Ultrastructural localization of the small GTP-binding protein Rap1 in human platelets and megakaryocytes. *Br J Haematol* 1994; 88: 372–382.
49 Shirakawa R, Yoshioka A, Horiushi H, Nishioka H, Tabuchi A, Kita T. Small GTPase rab4 regulates Ca^{2+}-induced alpha-granule secretion in platelets. *J Biol Chem* 2000; 275: 33844–33849.
50 Karniguian A, Zahraoui A, Tavitian A. Identification of small GTP-binding rab proteins in human platelets: thrombin induced phosphorylation of rab3B, rab6, and Rab8 proteins. *Proc Natl Acad Sci USA* 1993; 90: 7647–7651.
51 Heijnen HF, Debili N, Vainchencker W, Breton-Gorius J, Geuze HJ, Sixma JJ. Multivesicular bodies are an intermediate stage in the formation of platelet alpha-granules. *Blood* 1998; 91: 2313–2325.
52 Alberio L, Safa O, Clemetson KJ, Esmon CT, Dale GL. Surface expression and functional characterization of alpha-granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin. *Blood* 2000; 95: 1694–1702.
53 Taraboletti G, Roberts D, Liotta LA, Giavazzi R. Platelet thrombospondin modulates endothelial cell adhesion, motility, and growth: a potential angiogenesis regulatory factor. *J Cell Biol* 1990; 111: 765–772.
54 Clezardin P, Malaval L, Morel MC et al. Osteonectin is an alpha-granule component involved with thrombospondin in platelet aggregation. *J Bone Mineral Res* 1991; 6: 1059–1070.
55 Palabrica T, Lobb R, Furie BC et al. Leukocyte accumulation promoting fibrin deposition is mediated in vivo by P-selectin on adherent platelets. *Nature* 1992; 359: 848–851.
56 Marcondes S, Lafay M, Brohard-Bohn B, de Nucci G, Rendu F. Platelet induce human umbilical vein endothelial cell proliferation through P-selectin. *Life Sci* 2000; 66: 1817–1826.
57 Piccardoni P, Evanlegista V, Piccoli A, de Gaetano G, Walz A, Cerletti C. Thrombin-activated human platelets release two NAP-2 variants that stimulate polymorphonuclear leukocytes. *Thromb Haemost* 1996; 76: 780–785.
58 Brown KJ, Parish CR. Histidine-rich glycoprotein and platelet factor 4 mask heparan sulfate proteoglycans recognized by acidic and basic fibroblast growth factor. *Biochemistry* 1994; 33: 13918–13927.
59 Toyama-Sorimachi N, Kitamura F, Habuchi H, Tobita Y, Kimata K, Miyasaka M. Widespread expression of chondroitin sulfate-type serglycins with CD44 binding ability in haematopoietic cells. *J Biol Chem* 1997; 272: 26714–26719.
60 Rosenberg RN, Baskin F, Fosmire JA et al. Altered amyloid protein processing in platelets of patients with Alzheimer disease. *Arch Neurol* 1997; 54: 139–144.
61 Raccuglia G. Gray platelet syndrome. A variety of qualitative platelet disorders. *Am J Med* 1971; 51: 818–828.
62 Levy-Toledano S, Caen JP, Breton-Gorius J et al. Platelet syndrome: α-granule deficiency. Its influence on platelet function. *J Lab Clin Med* 1981; 98: 831–848.
63 Gerrard JM, Phillips DR, Rao GHR. Biochemical studies of two patients with the gray platelet syndrome. Selective deficiency of platelet alpha granules. *J Clin Invest* 1980; 66: 102–109.
64 Rendu F, Nurden AT, Lebret M, Caen JP. Futher investigations on Bernard–Soulier platelet abnormalities. A study of 5-hydroxytryptamine uptake and mepacrine fluorescence. *J Lab Clin Med* 1981; 97: 689–699.
65 Rosa JP, George JN, Bainton DF, Nurden AT, Caen JP, McEver RP. Gray platelet syndrome: demonstration of alpha granule membranes that can fuse with the cell surface. *J Clin Invest* 1987; 80: 1138–1146.
66 Hayward CP, Cramer EM, Kane WH et al. Studies of a second family with the Quebec platelet disorder: evidence that the degradation of the alpha-granule membrane and its soluble contents are not secondary to a defect in targeting proteins to alpha-granules. *Blood* 1997; 89: 1243–1253.
67 Bentfeld-Barker ME, Bainton DF. Identification of primary lysosomes in human megakaryocytes and platelets. *Blood* 1982; 59: 472–481.
68 Metzelaar MJ, Clevers HC. Lysosomal membrane glycoproteins in platelets. *Thromb Haemost* 1992; 68: 378–382.
69 Israels SJ, McMillan EM, Robertson C, Singhory S, McNicol A. The lysosomal granule membrane protein, LAMP-2, is also present in dense granule membrane. *Thromb Haemost* 1996; 75: 623–629.
70 Dangelmaier CA, Holmsen H. Determination of acid hydrolases in human platelets. *Anal Biochem* 1980; 104: 182–191.
71 Hoxie JA, Ahuja M, Belmonte E, Pizarro S, Parton R, Brass LF. Internalisation and recycling of activated receptors. *J Biol Chem* 1993; 268: 13756–13763.
72 Ciferri S, Emiliani C, Guglielmini G, Orlacchio A, Nenci GG, Gresele P. Platelets release their lysosomal content in vivo in humans upon activation. *Thromb Haemost* 2000; 83: 157–164.
73 Dell'Angelica EC, Mullins C, Caplan S, Bonifacio JS. Lysosome-related organelles. *FASEB J* 2000; 14: 1265–1278.

Cambridge Books Online © Cambridge University Press, 2009

8

Platelet receptors for thrombin

Lawrence F. Brass[1], Marina Molino[2], Peter J. O'Brien[1] and Mark Kahn[1]

[1]Departments of Medicine and Pharmacology, and the Center for Experimental Therapeutics at the University of Pennsylvania, Philadelphia, USA and the [2]Istituto di Ricerche Farmacologiche Mario Negri, Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy

Introduction

Thrombin is one of the most potent agonists that platelets will encounter in vivo, but unlike most of the others it is a protease. For years after thrombin was shown to be a platelet activator as well as an effector in the clotting cascade, the precise mechanism by which it activates platelets remained obscure. Binding studies demonstrated high affinity interactions with several sites on the platelet surface, including glycoprotein (GP) Ibα, but efforts to establish that any of these constituted a receptor in the signalling sense were not entirely successful. Substrates on the platelet surface for proteolytic cleavage by thrombin were also identified, including GP V, but cleavage of these sites did not appear to be required for platelet activation by thrombin. Before discussing the receptors that have been identified, it is worth considering what some of the criteria might be for establishing a protein as a true signalling receptor for thrombin. Such criteria would include (i) demonstrating its presence on the platelet surface, (ii) showing that it was a substrate for thrombin or closely associated with a substrate for thrombin, (iii) demonstrating an association of the candidate receptor with mediators or effectors for intracellular signalling cascades, (iv) showing that expression of the candidate receptor could render a cell that was otherwise unresponsive to thrombin capable of responding, and (v) showing that blocking, dismantling or otherwise removing the candidate receptor would reduce platelet responses to thrombin.

Clues to the identity of receptors that might meet these criteria were obtained in the late 1980s when several laboratories independently demonstrated that thrombin responses in human platelets were at least partially mediated by heterotrimeric G proteins. However, credit for the first identification of thrombin-responsive G protein coupled receptors goes to the Coughlin[1] and Pouyssegur[2] laboratories for their descriptions of protease-activated receptor-1 or PAR1. To date, PAR family members are the only receptors that have been described that meet all of the above criteria for mediating thrombin responses in platelets. However, it is not yet clear (as of late 2000) whether PAR family members are necessarily the only thrombin receptors on platelets or whether other proteins on the platelet surface play a role as well, either by initiating signalling themselves or by facilitating the activation of PARs.

The intention of this chapter is to review current information about the molecular basis for platelet activation by thrombin, with a particular emphasis on PAR family members. The first section will briefly describe the effects of thrombin on platelets. The second will summarize current knowledge about the four known members of the PAR family, particularly those expressed on human and mouse platelets. The third section will address the biology of PAR family members in platelets, while the final section will return to the potential role of other platelet receptors for thrombin. The reader is referred to chapters elsewhere in this volume dealing with related aspects of platelet biology.

Platelet responses to thrombin

Platelets are activated at sites of vascular injury by the combined effects of a number of molecules, including thrombin, collagen, von Willebrand factor, ADP, thromboxane A_2 and epinephrine. The relative contribution of each of these varies during the evolution of the hemostatic plug. Initially, newly exposed collagen fibrils and von Willebrand factor play the primary role, but as the first monolayer of adherent platelets forms, recruitment of non-adherent platelets

by locally generated thrombin, secreted ADP and released TxA_2 takes on increasing importance. Thrombin is generated from prothrombin because of the exposure of tissue factor, the activation of factors VII and IX, and the conversion of factor X to Xa to form the prothrombinase complex. Thrombin is able to activate platelets at concentrations as low as 0.1 nM (approximately 0.01 units per ml). Hallmarks of the platelet response to thrombin include reorganization of the actin cytoskeleton, secretion of the contents of the platelet's storage granules, exposure of fibrinogen receptors on the integrin $\alpha_{IIb}\beta_3$, and platelet aggregation. Underlying these responses is the activation of signalling pathways whose known effectors include phospholipase C, phospholipase A_2, and PI 3-kinase. Phospholipase C hydrolyses membrane phosphatidylinositol-4,5,-bisphosphate (PIP_2) to form 1,4,5–IP_3 and diacylglycerol, which in turn raise the cytosolic free Ca^{2+} concentration and activate protein kinase C isoforms. PLA_2 mobilizes arachidonic acid from platelet membranes, making it available as a substrate for COX-1 and conversion to TxA_2, itself a potent platelet agonist. Although other platelet agonists can also cause phosphoinositide hydrolysis, none appear to be as efficiently coupled to phospholipase C as thrombin. Within seconds of the addition of thrombin, the cytosolic Ca^{2+} concentration increases tenfold to approximately 1 μM, triggering downstream Ca^{2+}-dependent events, including the activation of phospholipase A_2. Thrombin also activates the Ras superfamily members such as Rac, Rho and Rap1B, leading to rearrangement of the actin cytoskeleton and shape change. Finally, thrombin, like ADP and epinephrine, is able to inhibit adenylyl cyclase activity in platelets, relieving the tonic inhibitory state caused by elevated cAMP levels in circulating platelets. Since small changes in 'basal' cAMP levels can have a large effect on the magnitude of platelet responses to agonists, suppression of cAMP formation plays an important role in platelet activation by thrombin.

Most, if not all, of these responses to thrombin are thought to be mediated by G proteins. Mammalian G proteins are commonly denoted according to the identity of their α subunit. G_α binds GTP in the on state and GDP in the off state. Human platelets express members of the G_q, G_{12}, and G_s family of G proteins, and at least four members of the G_i family, G_{i1}, G_{i2}, G_{i3} and G_z. Biochemical and gene deletion studies suggest that G_q is the primary link to phospholipase Cβ activation in platelets[3], with an incompletely defined contribution from G_i family members. As in other cells, G_s and the three G_i family members, respectively, stimulate and inhibit adenylyl cyclase activity in platelets, and, in the case of the G_i family members probably do other things as well. The G_{12} family members in platelets (G_{12} and G_{13}) are believed to regulate the actin cytoskeleton and, therefore, shape change[4,5]. Of these G proteins, there is indirect evidence that thrombin signalling involves G_q, G_i and G_{12} family members, although recent evidence suggests that thrombin's ability to inhibit cAMP formation is largely mediated by secreted ADP[6], despite the fact that PAR1 can couple to G_i family members in reconstitution systems[7].

Biology of the PAR family of receptors

Four PAR family members have been identified to date. Three (PAR1, PAR3 and PAR4) are thrombin receptors. The fourth, PAR2, is activated by serine proteases other than thrombin (Table 8.1). Each of these is composed of a single polypeptide with an extracellular N-terminus, an intracellular C-terminus and seven membrane-spanning domains – a basic structure that is common to nearly all of the known G protein coupled receptors (Fig. 8.1). PAR1 was initially identified using a cloning strategy with RNA derived from thrombin-responsive cells and of the four current family members it is still the one about which there is the most known[1,2]. It is also the one that established the activation paradigm that in general terms applies to the other three family members[1]. In each case, receptor activation occurs when the appropriate protease binds to the extended N-terminus of the receptor, cleaving it and exposing a new N-terminus that serves as a tethered ligand for the receptor. The binding site for the tethered ligand is presumed to be formed by the extracellular loops of the 'body' of the receptor. Because the ligand is not free to diffuse away from the receptor, it is thought to present a high effective local concentration at the receptor and, perhaps, maintain signalling longer than would otherwise occur. As is the case for other G protein coupled receptors, contact between PAR ligands and the receptor is thought to initiate signalling because of an induced conformational change in the receptor that is transmitted across the plane of the plasma membrane to promote exchange of GTP for GDP on associated G proteins. In the case of PAR family members, the activation paradigm that was initially established for PAR1 includes the ability to respond to peptides based upon the sequence of the tethered ligand. The one exception to the rule is PAR3 for which no activating peptide agonist has yet been reported.

In contrast to PAR1, PAR2 was cloned serendipitously and only subsequently found to be activated by trypsin[8–10], tryptase[15–17]. PAR3 was identified as a second thrombin receptor after gene ablation studies showed that platelets from mice lacking PAR1 were still responsive to throm-

Cambridge Books Online © Cambridge University Press, 2009

Table 8.1. PAR family members[a]

	PAR1	PAR2	PAR3	PAR4
Present on human platelets?	Yes	No	?[50] and see text	Yes
Present on mouse platelets?	No	No	Yes	Yes
Activating Proteases	Thrombin Trypsin Xa Granzyme A[b]	Tryptase Trypsin Xa TF/VIIa MT-SP1[d]	Thrombin[f]	Thrombin Trypsin Cathepsin G[f]
Inactivating proteases	Plasmin, Elastase, Cathepsin G, Proteinase 3[e]	–	–	–
Cleavage sequence and tethered ligand	LDPR/*SFLLR*	SKGR/*SLIGK*	LPIK/*TFRGAP*	PAPR/*GYPGQV*
Examples of peptide agonists[c]	SFLLRN TFLLRN GFIYF	SLIGKV SFLLRN	None known	GYPGKF AYPGKF[60]
Consequences of gene deletion in mice	Partial embryonic lethality; no hemostatic abnormalities in surviving knockout mice[18,93]	Mild impairment of leukocyte migration; no embryonic loss[94]	Loss of thrombin signalling in platelets at low thrombin concentrations; no embryonic loss[21]	Not yet reported

Notes:

[a] The references included in this table are not intended to be exhaustive. See text as well.

[b] Activating proteases for PAR1: Thrombin[1,2]. Trypsin[1,22,38]. Factor Xa[95,96]. Granzyme A[97,98]

[c] Structure/activity relationships have been explored in some detail for short peptides (up to 14 amino acids) that activate PAR1 and PAR2[28,99–106], and more recently for PAR4[21,51,60,107]. To date, no peptide activators of PAR3 have been described[108].

[d] Optimal activation of PAR2 by factor VIIa appears to require co-expression of tissue factor (TF) and the presence of factor X[16]. Under these conditions the concentration of factor VIIa that is required to elicit signalling through PAR2 drops to 8 pM (EC50). This basic phenomenon was observed in several mammalian cell lines, human umbilical vein endothelial cells and injected *Xenopus laevis* oocytes (see references below), although not in baby hamster kidney (BHK) cells[109]. It does not require the cytoplasmic domain of tissue factor[16,110]. Tryptase is able to cleave and activate PAR2, but with a potency less than trypsin. Trypsin[8–14]. Tryptase[11–14]. Factor Xa[15,16]. TF/VIIa[16,17]. MT-SP1[111].

[e] Inactivating proteases for PAR1: Plasmin[15,38], cathepsin G[72,112], elastase[112], and proteinase 3[112].

[f] Activating proteases for PAR3: Thrombin[18,19]. Activating proteases for PAR4: Thrombin[21], Trypsin[21], Cathepsin G[74].

bin[18]. It is a major regulator of thrombin responses in rodent platelets[19], but little else is known about it. When overexpressed, human PAR3 can respond to thrombin by a mechanism that was initially thought to resemble PAR1. However, there are as yet no well-studied examples of signalling by PAR3 in human tissues and recent evidence suggests that on murine platelets PAR3 serves solely to facilitate cleavage of PAR4 by thrombin[20]. The fourth family member, PAR4, was identified by database searches using conserved domains of the other three family members[21,22]. PAR4 is expressed on human and mouse platelets and presumably accounts for the continued ability of platelets from PAR3 knockout mice to respond to thrombin[21,22].

Thus, the four PAR family members have some features in common, but there are differences among them as well. As noted above, three of the receptors can be activated by thrombin. Two of these, PAR1 and PAR3, have similar dose–response curves when studied in expression systems. The third, PAR4, requires 10–100-fold higher concentrations of thrombin, apparently because it lacks the hirudin-like sequences that can interact with thrombin's anion-binding exosite and facilitate receptor cleavage[20–23]. This distinction is important for understanding the role of PAR4 in human and mouse platelets and will be discussed further later in this chapter.

Cambridge Books Online © Cambridge University Press, 2009

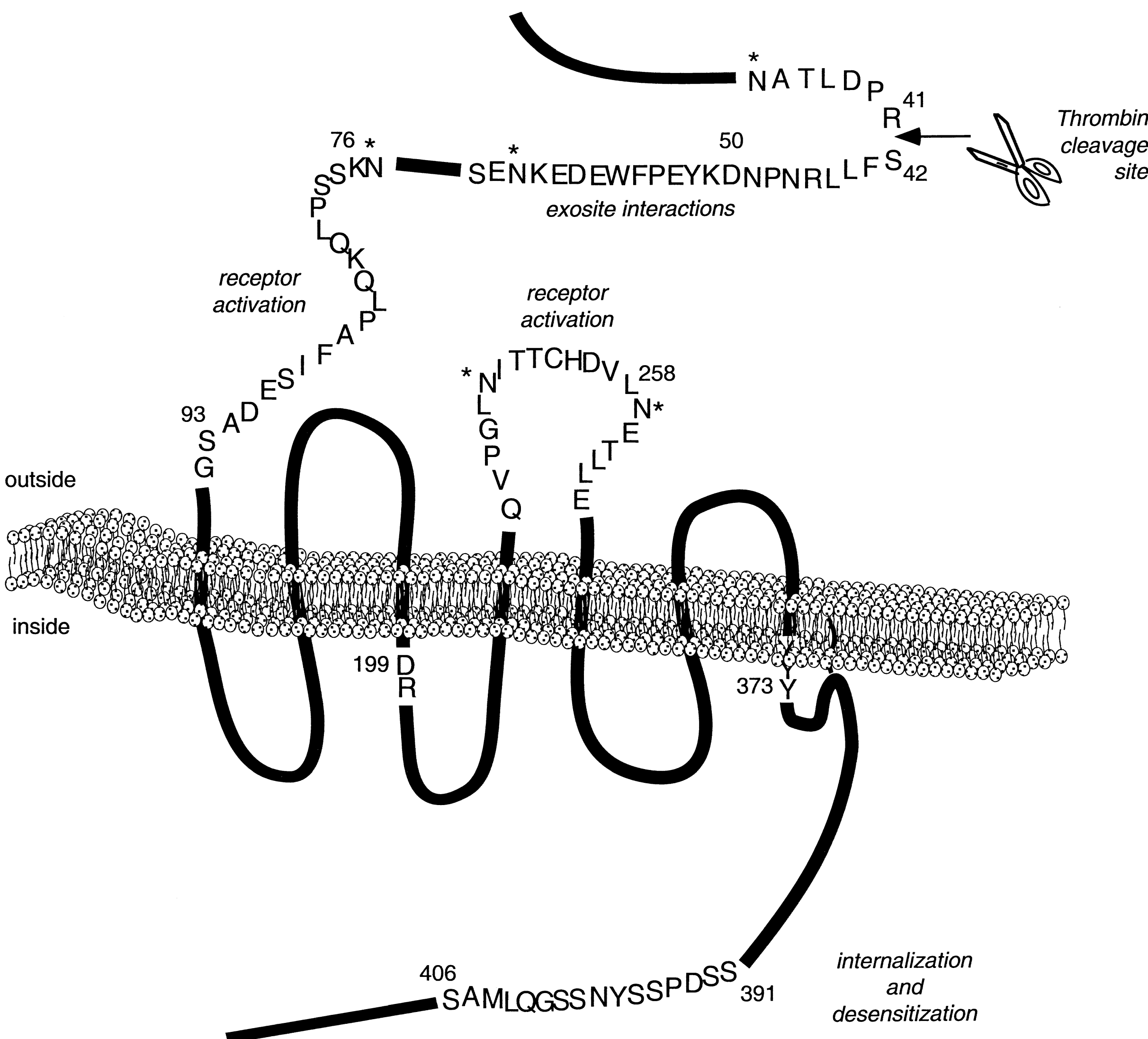


Fig. 8.1. Structure and features of PAR1. Cartoon of human PAR1 highlighting domains thought to be involved in receptor activation and interactions with thrombin. Additional details and references are included in the text. Potential sites for N-linked glycosylation are marked with an asterisk. L258P, D199R/R200D and Y371A/Y372A/Y373A are some of the known induced mutations that will inactivate PAR1. There are (as yet) no known naturally occurring PAR1 mutants that result in receptor inactivation.

Genetics and tissue distribution

The genes encoding all four PAR family members have a similar structure with a single intron interrupting the sequence encoding the receptor N-terminus. Their genetic loci are conserved across species, suggesting a common ancestral origin[24]. In humans PAR1, PAR2, and PAR3 are tightly clustered at chromosome 5q13, while PAR4 is located separately at 19p12[22,24]. In mice PAR1, PAR2 and PAR3 are in a cluster that maps to 13d2. Murine PAR4 is located at 8b3[24]. If genes for additional PAR family members exist, they have yet to be identified. Information about the tissue distribution of the four PAR family members is most complete for PAR1 and PAR2 since antibodies for these receptors are widely available. PAR1 and PAR2 are found in a variety of tissues and cell lines, and have been described in several species and during embryonic development (recently reviewed in[25], see also reference[26]). The patterns of expression of PAR3 and PAR4 are less well characterized, and rely primarily upon analyses of mRNA expression. While the message for PAR3 appears to

Cambridge Books Online © Cambridge University Press, 2009

be widely distributed in human tissues, murine PAR3 mRNA is highly expressed only in megakaryocytes, bone marrow, and spleen[19]. Human PAR4 mRNA is abundant in gastrointestinal tissues including the liver, small intestine, and pancreas, as well as in megakaryocytes, lung, placenta, thyroid, and prostate tissues[21,22]. Whether the receptor is actually expressed in those tissues remains to be demonstrated.

Identification of the ligand binding domain

Characterization of the ligand binding domain for PAR tethered ligand domains is most complete for PAR1 and PAR2. These receptors are approximately 30% identical to each other with near-identity of sequence within the second extracellular loop (ECL2), a region implicated in ligand docking[9,27,28]. The first studies of the PAR1 ligand binding domain used antibodies to inhibit activation of the receptor by thrombin and the peptide agonists, SFLLRN. Two different polyclonal antibodies were used, one raised against the entire receptor N-terminus, the other raised against a 20-residue peptide spanning the thrombin cleavage site[29,30]. The latter antibody prevented PAR1 activation by thrombin, but not by SFLLRN. The former blocked PAR1 activation by both, suggesting that it might target the ligand binding site. Follow-up studies showed that the antiserum raised against the entire N-terminus contains antibodies to the membrane-proximal region (residues 83–94), implicating this domain in ligand binding[30] (Fig. 8.1).

In subsequent studies, the ligand binding domain was mapped using chimeras between human and *Xenopus* PAR1[31,32]. Those studies also highlighted a region of the N-terminus near the first transmembrane domain, as well as the second extracellular loop. Selected point mutations suggested that a residue in the membrane-proximal amino terminal exodomain (Phe 87) and a residue in ECL2 (Glu 260) are sufficient to confer selectivity for the PAR1 agonist peptides. The interaction of ECL2 with the SFLLRN ligand peptide was determined to occur between Glu260 and Arg46 in the tethered ligand domain, and to a lesser extent Phe87 in the membrane-proximal amino terminal exodomain. Nanievicz and coworkers[33] also found that insertion of eight residues of *Xenopus* ECL2 into human PAR1 produces a constitutively active receptor. In a similar approach, Lerner et al.[27] created chimeric gain-of-function receptors to determine which receptor domains specify agonist selectivity. Again, the results suggest that ECL2 confers most of the agonist selectivity. An additional interaction between the amino-terminal exodomain and the third extracellular loop (ECL3) was also demonstrated. Taken together, the chimera studies suggest that the extracellular domains of protease-activated receptors interact to form a ligand binding site that is critical for signalling.

Inactivation and replacement of PAR family members

Once activated by a protease, PAR family members need to be turned off to prevent indefinite signalling. Since cleaved receptors are unable to respond to proteases a second time, cleaved receptors also need to be replaced in order to permit a second round of responses to the protease. How this is accomplished varies among the family members and can be different in different types of cells. Several common mechanisms for terminating signalling through other G protein coupled receptors also apply to PAR family members. These include receptor desensitization, which uncouples receptors from their G proteins; receptor endocytosis, which removes cleaved receptors from the cell surface; and receptor downregulation, which leads to a reduction in total receptor number[34]. In some cases, proteolysis of the tethered ligand domain may also play a role[35].

Indirect evidence for the role of receptor phosphorylation in PAR1 signal termination was first obtained in studies of megakaryoblastic cell lines when it was shown that the recovery of signalling through receptors that had been activated by peptide agonists could be retarded by adding inhibitors of serine/threonine phosphatases[36]. Proof that phosphorylation of PAR1 actually occurs was obtained by Van Obberghen-Schilling and coworkers using an epitope-tagged PAR1 construct expressed in CCL39 cells[37,38]. Ishii et al.[39] later showed that coexpression of PAR1 with the kinase, GRK-3 (βARK-2), in *Xenopus laevis* oocytes selectively inhibits PAR1 signalling. This was later demonstrated in vivo, when transgenic mice overexpressing GRK-3 were shown to have significantly attenuated thrombin signalling[40]. Truncation of the C-terminus of PAR1 at residue 397 or replacement of all of the serine and threonine residues with alanine renders PAR1 insensitive to GRK-3 regulation (see Fig. 8.1 and[39]). These alanine mutants and truncated receptors signal more robustly than their wild type counterparts, as might be predicted if phosphorylation were linked to receptor inactivation[39,41,42]. Finally, GRK family members may not be the only kinases capable of phosphorylating PAR1. Ido et al. have recently identified a 33 kDa kinase capable of phosphorylating the C-terminus of PAR1 fused to GST[43]. As yet, little is known about the role of this kinase in regulating PAR1 signalling in cells that normally express the receptor.

The carboxyl tail of PAR1 also participates in receptor

Cambridge Books Online © Cambridge University Press, 2009

shutoff through its role in agonist-induced receptor internalization and trafficking[44–46] (Fig. 8.1). For many G protein coupled receptors, internalization can contribute to receptor resensitization. PAR family members are an exception to this rule since once cleaved, they cannot be activated a second time by a protease. Information about the internalization and trafficking of PAR1 and, to a lesser extent, PAR2 has been obtained by several groups of investigators[34,44,47–49]. In most types of cells, PAR1 and PAR2 are rapidly internalized after agonist stimulation. In megakaryoblastic cell lines, ~85% of PAR1 is sequestered into coated pits and then endosomes within one minute of activation[47]. In endothelial cells, fibroblasts, and epithelial cells, the internalization of PARs is not as complete, with rapid internalization of at most ~60% of cell surface receptors. Most of the internalized receptors are transferred to lysosomes, but the sorting is not completely efficient and in megakaryoblastic cell lines in particular as much as 40% of the cleaved receptors are recycled back to the cell surface[47]. The physiological relevance of this recycling is unknown. It does not appear to occur on platelets.

PARs and platelets

Given all of this information about protease-activated receptors, how does it all fit together for platelets and can the known members of the PAR family account for everything that is known about platelet responses to thrombin? Are there additional receptors on the platelet surface that contribute either directly or indirectly to the initiation of platelet activation by thrombin? These are the questions that will be considered in the last two sections of this chapter. On the face of it, the answers to these questions seem straightforward. It was shown nearly 10 years ago that human platelets express PAR1 and that the addition of PAR1 agonist peptides such as SFLLRN would mimic thrombin's ability to cause platelet aggregation and secretion[1]. There were hints from studies with PAR1 blocking antibodies and with platelets from some non-primate species that a second thrombin receptor might also be present, but definitive evidence for this came much later with the knockout of PAR1 in mice[18] and the identification of PAR3[19] and PAR4[21,22]. Of the three known thrombin-responsive members of the PAR family, human platelets clearly express PAR1 and PAR4. Agonist peptides for either will activate human platelets[1,21]. PAR3 has been detected on human platelets and endothelial cells[50], but so far there is no evidence that it contributes to thrombin responses in either. Not only is there no known agonist peptide that activates human PAR3, but simultaneous inhibition of PAR1 and PAR4 with blocking antibodies or a small molecule antagonist (in the case of PAR1) completely abolishes human platelet responses to thrombin[51]. This would not, of course, rule out the possibility that PAR3 facilitates activation of human PAR4 as it does in mice. However, human PAR3 appears to signal more readily in response to thrombin than does mouse PAR3, so the failure to detect a signal attributable to PAR3 in platelets and endothelial cells is harder to explain (discussed further in references[52,53]).

Thus, the picture has emerged that thrombin responses in human platelets are largely, if not entirely, mediated by PAR1 and PAR4 (Fig. 8.2). Cleavage of human PAR4 requires a higher concentration of thrombin than does cleavage of PAR1 and it is likely that PAR1 is the predominant signalling receptor at low thrombin concentrations. Recent evidence suggests that PAR4 not only kicks in at higher thrombin concentrations, but also produces a more sustained signal[54,55]. The potential physiological significance of this remains to be established in human platelets, although it may account for the results from a number of studies that suggested that PAR1 agonist peptides do not have quite the same range of effects on platelets as does thrombin (e.g. references[56–59]). It has recently been shown that human PAR4 is incapable of inhibiting cAMP formation by adenylyl cyclase, which suggests that it is not coupled to the G_i family members that are present in human platelets[60]. On the other hand, as already noted, it also appears that human PAR1, which is capable of inhibiting adenylyl cyclase in reconstitution systems, may not do so in intact human platelets. Therefore, the significance of this difference between PAR1 and PAR4 also needs to be examined further. Otherwise, current evidence suggests that PAR1 and PAR4 are both coupled to G_{12} to cause shape change, while G_q and possibly G_i activate phospholipase Cβ. The link from G_i to phospholipase C is suggested by old data which showed that in platelets permeabilized with saponin and in megakaryoblastic cell lines that express PAR1, thrombin-induced phosphoinositide hydrolysis is at least partially sensitive to pertussis toxin[61,62]. Pertussis toxin ADP-ribosylates and uncouples the α subunits of all of the G_i family members in human platelets except for G_z. Since platelets (unlike most cells) are impermeable to pertussis toxin, entry can only be gained by using agents such as saponin which create gaps in the platelet plasma membrane by sequestering cholesterol – and at the same time perturbing the membrane to the point where the outcome of the experiment may be affected.

Mice are different

Mouse platelets also express two PAR family members, but they are PAR3 and PAR4, rather than PAR1 and PAR4. The

Cambridge Books Online © Cambridge University Press, 2009

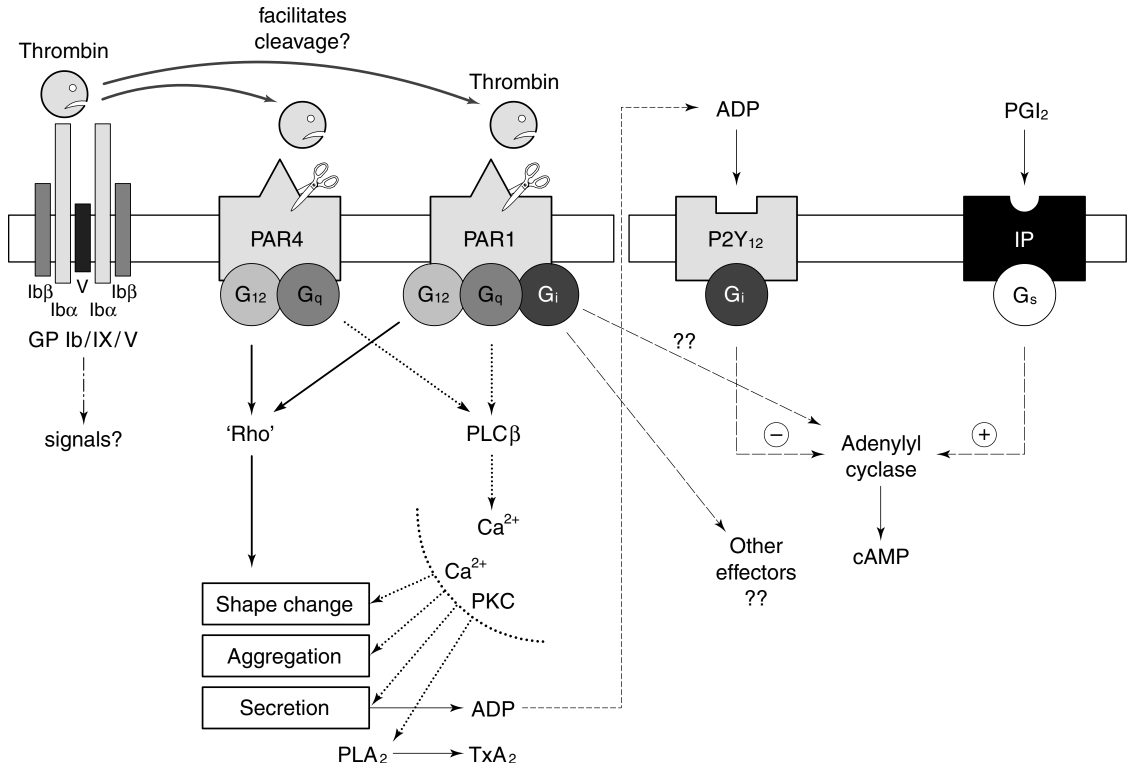


Fig. 8.2. Thrombin signalling in human platelets. Human platelets express PAR1 and PAR4. and, in addition, have a high affinity binding site for thrombin on the GP Ib/IX/V complex. They may also express PAR3, although the functional significance of any PAR3 on human platelets (unlike mouse platelets) is unclear. GP V is a substrate for thrombin. Thrombin can cleave and activate PAR1 and PAR4. The binding site on GP Ibα may facilitate this process. Once receptor activation has occurred, heterotrimeric G proteins serve as mediators to couple the receptors to intracellular effectors, leading to shape change, aggregation and secretion.

PAR1 knockout made it clear that the failure of mouse platelets to respond to PAR1 agonist peptides was due to the absence of PAR1 from mouse platelets. The subsequent identification of PAR3 and PAR4, and the knockout of PAR3 has led to the model that although murine platelets express both of the these receptors, signalling is due solely to PAR4[20] (Fig. 8.3). The role of PAR3 on murine platelets appears to be to facilitate the cleavage of PAR4 at low thrombin concentrations. This is necessary because the N-terminus of PAR4 lacks a domain that can interact with the anion-binding exosite on thrombin. This exosite-interacting is present on PAR1, but not on PAR4 (Fig. 8.1). On the face of it, this seems an awkward arrangement, since it implies that on mouse platelets thrombin binds first to PAR3, cleaves it (without generating a signal), and then cleaves a physically proximate PAR4. Supporting this conclusion is the PAR3 knockout, which shifts the dose/response curve for platelet activation by thrombin to higher concentrations, and the inability of mouse PAR3 to signal efficiently in expression systems[20,21]. If the model is correct, then platelets from mice lacking PAR4, but not lacking PAR3, should be unresponsive to thrombin. Evidence that this is the case has just been reported[113].

Expression levels and the location of thrombin receptors on platelets

In resting human platelets, PAR1, like many other platelet surface proteins, seems to be distributed in two compartments. The first is on the exposed platelet plasma membrane. The second is on the surface connecting membrane system, which evaginates and supplies additional membrane for the platelet surface when platelets are activated and change shape. Based on binding studies with a monoclonal antibody specific for human PAR1, there are approximately 2000 copies of PAR1 on the surface of resting platelets, plus an additional 40% more in the surface connecting system which is externalized when platelets are activated and change shape[63–65]. There is no information on PAR4. Notably, the number of copies of PAR1 detected

Cambridge Books Online © Cambridge University Press, 2009

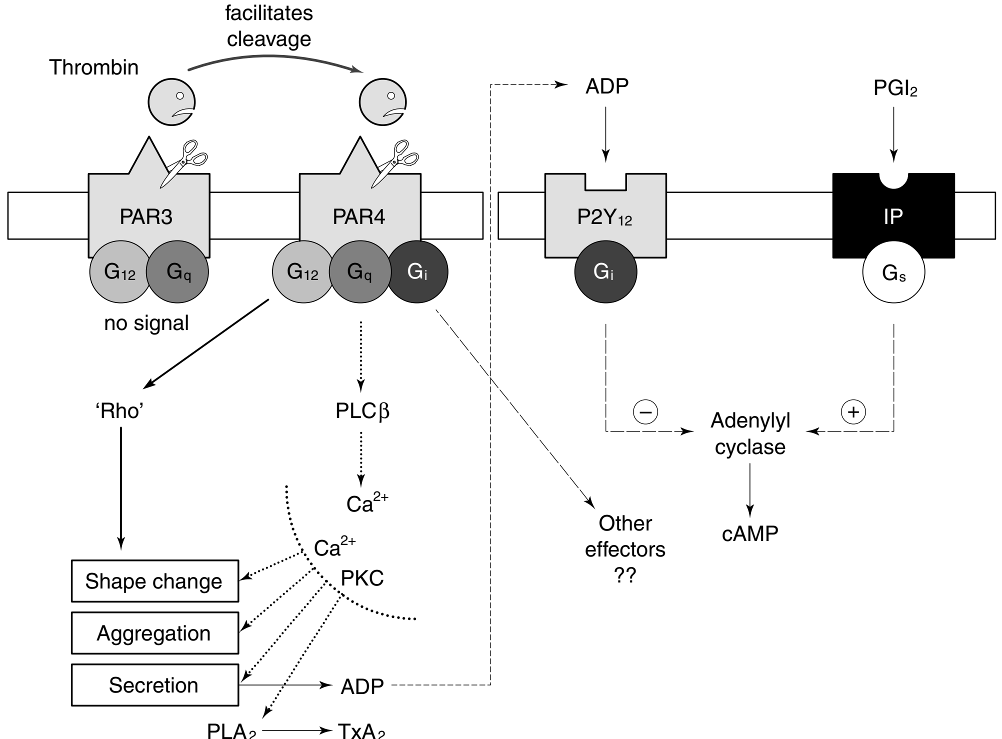


Fig. 8.3. Thrombin signalling in mouse platelets. Mouse platelets express PAR3 and PAR4, but not PAR1. Present evidence suggests that the PAR3 facilitates cleavage of PAR4 at low thrombin concentrations, but does not itself cause signalling.

in the antibody binding studies (a few thousand), fits reasonably well with the number of moderate affinity ^{125}I-thrombin binding sites identified by Harmon and Jamieson[66,67] prior to the cloning of PAR1. It is likely that PAR1 and PAR4 are clustered in membrane microdomains such as rafts, but to our knowledge, this issue has not been directly addressed. There is also no information yet available about the level of expression and distribution of PAR3 and PAR4 on mouse platelets. Indirect evidence from over-expression studies[68] and from studies on endothelial cells[52] suggests that PAR family members can be sufficiently close to each other to allow transactivation of PAR1 or PAR2 by cleaved PAR1. That supports the concept that PAR3 and PAR4 can reside close enough to each other for thrombin bound to murine PAR3 to be able to cleave PAR4 – as does the mounting evidence that other G protein coupled receptors can physically associate with one another. However, this remains to be directly established.

Cleavage and recycling of thrombin receptors on human platelets

Here again, most of the available information is from studies on PAR1. Cleavage of PAR1 can be detected fairly easily as a loss of binding sites for monoclonal antibodies whose epitopes include the cleavage site and therefore, are lost when the receptor is cleaved by thrombin[69]. Cleavage can also be detected with assays for the released N-terminal fragment[70,71]. The results obtained through both approaches suggest that thrombin rapidly cleaves most, if not all, of the copies of PAR1 that are present on resting platelets, as well as those that become exposed as the platelets activate. Rapidly in this case means within seconds following the addition of even moderate thrombin concentrations. The cleaved fragment can be recovered quantitatively in the fluid phase, suggesting that none of it is retained on the platelet surface.

Once cleaved by thrombin, PAR1 on human platelets appears to be subject to three possible fates. Most of it remains on the surface in a cleaved state, but some is internalized and some is shed into membrane microvesicles[65]. This is different from other cells that express PAR1, such as

Cambridge Books Online © Cambridge University Press, 2009

fibroblasts or endothelial cells. As was described earlier in this chapter, activated PAR1 on cells other than platelets is rapidly internalized into an endosomal compartment and then either recycled or (more commonly) delivered to lysosomes where it is degraded. Replacement occurs by the movement of intact receptors to the cell surface, either directly or after temporary storage in an intracellular reserve pool. So far, there is no information about the fate of activated PAR4 in platelets. Since platelets do not normally encounter thrombin more than once, it is not entirely surprising that they lack the necessary cellular machinery to fully remove and replace cleaved thrombin receptors.

Interactions of platelet protease-activated receptors with proteases other than thrombin

An extensive literature exists on the interaction of PAR family members with proteases other than thrombin (reviewed in reference[53]). Much of it concerns PAR1. Summarizing, the results suggest that proteases that uniquely cleave PAR1 at the same site as thrombin will expose the tethered ligand domain and can cause receptor activation. Proteases that cleave elsewhere in the N-terminus potentially remove the tethered ligand domain, rendering the receptor unresponsive to thrombin, although not necessarily unresponsive to an agonist peptide. Trypsin, factor Xa and granzyme A have been shown to be able to cleave and activate PAR1, although not necessarily with the same rapid kinetics as thrombin (see Table 8.1). Their physiological relevance for platelets is not yet clear. Examples of proteases that can disable signalling through PAR1 include plasmin and two neutrophil proteases, cathepsin G and elastase (see Table 8.1). These proteases might be expected to accumulate at sites of vascular thrombosis or inflammation, placing them at sites where they could influence PAR1 cleavage by thrombin. Whether this occurs in vivo still remains to be established. The site of PAR1 cleavage by cathepsin G lies between residues Phe55 and Trp56[72]. Cathepsin G can also inactivate PAR3[73]. The paradoxical ability of cathepsin G to cause human platelet activation is apparently explained by activation of PAR4[74].

Other potential thrombin receptors on platelets

Long before the identification of PAR1, evidence was obtained by the Jamieson laboratory that the extracellular domain of GP Ibα contains a high affinity binding site for thrombin[66,75]. Binding studies with radioiodinated thrombin suggested that this site had a K_D of approximately 0.1 nM and a B_{max} of 50–100 sites per platelet[66,75]. Although the apparent discrepancy between this number of sites and the much larger number of copies of GP Ibα (15 000–25 000 per platelet) has never been fully explained, the suggestion was made that GP Ib might be not only a thrombin binding site, but also a participant in thrombin-initiated platelet activation. This suggestion has persisted despite falling out of fashion for a period. Recent studies have revived the issue. GP Ib is a heterodimer composed of an α and a β subunit that are disulfide linked. It forms a complex with two other proteins, GP IX and GP V in a 2: 2: 1 (Ib: IX: V) ratio that serves a well-established role as a binding site for von Willebrand factor and as an anchor for the platelet cytoskeleton (reviewed in reference[76] and elsewhere in this volume). There is a binding site for thrombin located at approximately residues 268–287 on GP Ibα which is thought to interact with domains other than the active site[77]. Interestingly, when taken as a whole, the molecular weight of the entire GP Ib/IX/V complex (all five proteins) approaches that predicted by target size analysis of the thrombin binding site on platelets[67].

In addition to serving as a thrombin and vWF binding site, recent studies show that GP Ib/IX/V can initiate signalling in platelets and in heterologous systems (e.g. references[78–81]. Platelets from patients with the Bernard–Soulier syndrome lack GP Ib/IX/V and have a reduced response to thrombin – but they also have an aberrant morphology (reviewed in reference[76]). Deletion of the sialic acid rich 'glycocalicin' domain of GP Ibα removes the highest affinity thrombin binding site and blockade of the binding site with antibodies or lectins has been shown to decrease platelet responses to thrombin, particularly at low thrombin concentrations[75,82–84]. Dormann et al.[85] have recently provided evidence that the binding of thrombin to GP Ib is needed for platelets to fully express procoagulant activity and suggested that the lack of binding to GP Ibα may be one reason for the discrepancies between platelet responses to thrombin and SFLLRN, particularly with regard to the development of procoagulant activity (see for example references[56–59]). The existence of PAR4 is obviously another.

What is not clear from the evidence discussed so far is the mechanism by which thrombin binding to GP Ib might affect platelet responses to thrombin. There are at least two theoretical mechanisms. Thrombin, like von Willebrand factor, could initiate signalling through the cytoplasmic domains of GP Ib/IX/V. Alternatively, the binding of thrombin to GP Ibα could facilitate the cleavage of a PAR family member on human platelets, much as the binding of thrombin to PAR3 is thought to facilitate cleavage of PAR4

Cambridge Books Online © Cambridge University Press, 2009

on mouse platelets at low thrombin concentrations (Figs. 8.2 and 8.3). There is not yet enough evidence to firmly establish either of these possibilities. However, a recent study by De Candia et al.[84] shows that blockade of the interaction between thrombin and GP Ib impairs the cleavage of PAR1 on human platelets. The effect was an approximately fivefold reduction in the kcat/Km for PAR1 hydrolysis detected as the loss of the cleavage-sensitive binding site for a PAR1-directed monoclonal antibody. Furthermore, a report by Ramakrishnan et al.[86] suggests that cleavage of GP V by thrombin (a phenomenon first reported many years ago[87]) may release a suppressive effect on thrombin-induced platelet activation. Thus, although it is very clear that PAR1 and PAR4 provide the primary response elements for thrombin on human platelets, it remains possible that interactions with one or more members of the GP Ib/IX/V complex may facilitate cleavage/activation of PAR1 or otherwise regulate platelet activation by thrombin.

Conclusion: things are fitting together, but some pieces may still be missing

At this point it is fair to say that a lot has been learned about the molecular basis for thrombin responses in platelets, but some interesting issues remain to be resolved. There seems to be little question that the PAR family members expressed on human and mouse platelets are the primary receptors for thrombin. The precise contribution of the GP Ib/IX/V complex (discussed in the previous section) still remains to be settled and may not be resolved by studies on mouse platelets because of differences between mouse and human GP Ib. The mechanisms that limit the duration of signalling through thrombin receptors are arguably particularly important in preventing overly robust platelet activation at sites where it is not warranted. The role of receptor phosphorylation and uncoupling in that process still remains to be resolved, particularly since cleaved receptors tend to remain on the platelet surface and not be internalized. It has recently been shown that there are polymorphisms in the human PAR1 gene which may be associated with an increased risk of venous thrombosis[88], but not myocardial infarcts[89]. A polymorphism in the second extracellular loop of PAR2 has been shown to affect PAR2 activation by trypsin[90]. This issue needs further study, as does the reported ability of the released N-terminus of PAR1 to activate platelets[91,92]. In addition, there still remain a number of unsettled issues about signalling from these receptors. Precisely which G proteins are coupled to PAR1 and PAR4, and why does it appear that neither receptor is able to inhibit adenylyl cyclase via G_i in intact platelets? Are there signalling mechanisms involved in platelet responses to thrombin that do not immediately involve phospholipase C, adenylyl cyclase, PI 3-kinase and other known direct effectors of G protein α and $\beta\gamma$ subunits? Finally, are there additional thrombin-binding molecules on the surface of platelets that either signal directly in response to thrombin or modify the responses of known receptors to activation by thrombin? Although much is now known, a complete understanding of platelet activation by thrombin is still some time away.

References

1 Vu T-KH, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* 1991; 64: 1057–1068.

2 Rasmussen UB, Vouret-Craviari V, Jallat S et al. cDNA Cloning and expression of a hamster α-thrombin receptor coupled to Ca^{2+} mobilization. *FEBS Lett* 1991; 288: 123–128.

3 Offermanns S, Toombs CF, Hu YH, Simon MI. Defective platelet activation in $G\alpha_q$-deficient mice. *Nature* 1997; 389: 183–186.

4 Offermanns S, Laugwitz K-L, Spicher K, Schultz G. G proteins of the G_{12} family are activated via thromboxane A_2 and thrombin receptors in human platelets. *Proc Natl Acad Sci USA* 1994; 91: 504–508.

5 Klages B, Brandt U, Simon MI, Schultz G, Offermanns S. Activation of G_{12}/G_{13} results in shape change and Rho/Rho-kinase-mediated myosin light chain phosphorylation in mouse platelets. *J Cell Biol* 1999; 144: 745–754.

6 Kim S, Quinton TM, Cattaneo M, Kunapuli SP. Evidence for diverse signal transduction pathways in thrombin receptor activating peptide (SFLLRN) and other agonist-induced fibrinogen receptor activation in human platelets. *Blood* 2000; 96: 242a.(abstract)

7 Barr AJ, Brass LF, Manning DR. Reconstitution of receptors and GTP-binding regulatory proteins (G proteins) in Sf9 cells – a direct evaluation of selectivity in receptor. G protein coupling. *J Biol Chem* 1997; 272: 2223–2229.

8 Nystedt S, Emilsson K, Wahlestedt C, Sundelin J. Molecular cloning of a potential proteinase activated receptor. *Proc Natl Acad Sci USA* 1994; 91: 9208–9212.

9 Nystedt S, Emilsson K, Larsson AK, Strömbeck B, Sundelin J. Molecular cloning and functional expression of the gene encoding the human proteinase-activated receptor 2. *Eur J Biochem* 1995; 232: 84–89.

10 Nystedt S, Larsson A-K, Aberg H, Sundelin J. The mouse proteinase-activated receptor-2 cDNA and gene. *J Biol Chem* 1995; 270: 5950–5955.

11 Molino M, Barnathan ES, Numerof R et al. Interactions of

Cambridge Books Online © Cambridge University Press, 2009

mast cell tryptase with thrombin receptors and PAR-2. *J Biol Chem* 1997; 272: 4043–4049.

12 Corvera CU, Dery O, McConalogue K et al. Mast cell tryptase regulates rat colonic myocytes through proteinase-activated receptor 2. *J Clin Invest* 1997; 100: 1383–1393.

13 Schechter NM, Brass LF, Lavker RM, Jensen PJ. Reaction of mast cell proteases tryptase and chymase with protease activated receptors (PARs) on keratinocytes and fibroblasts. *J Cell Physiol* 1998; 176: 365–373.

14 Alm AK, Gagnemo-Persson R, Sorsa T, Sundelin J. Extrapancreatic trypsin-2 cleaves proteinase-activated receptor-2. *Biochem Biophys Res Commun* 2000; 275: 77–83.

15 Parry MAA, Myles T, Tschopp J, Stone SR. Cleavage of the thrombin receptor: Identification of potential activators and inactivators. *Biochem J* 1996; 320: 335–341.

16 Camerer E, Huang W, Coughlin SR. Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. *Proc Natl Acad Sci USA* 2000; 97: 5255–5260.

17 Camerer E, Rottingen J-A, Gjernes E et al. Coagulation factors VIIa and Xa induce cell signaling leading to up-regulation of the *egr-1* gene. *J Biol Chem* 1999; 274: 32225–32233.

18 Connolly AJ, Ishihara H, Kahn ML, Farese RV, Jr., Coughlin SR. Role of the thrombin receptor in development and evidence for a second receptor. *Nature* 1996; 381: 516–519.

19 Ishihara H, Connolly AJ, Zeng D et al. Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature* 1997; 386: 502–508.

20 Nakanishi-Matsui M, Zheng YW, Sulciner DJ, Weiss EJ, Ludeman MJ, Coughlin SR. PAR3 is a cofactor for PAR4 activation by thrombin. *Nature* 2000; 404: 609–610.

21 Kahn ML, Zheng YW, Huang W et al. A dual thrombin receptor system for platelet activation. *Nature* 1998; 394: 690–694.

22 Xu W-F, Andersen H, Whitmore TE et al. Cloning and characterization of human protease-activated receptor 4. *Proc Natl Acad Sci USA* 1998; 95: 6642–6646.

23 Ishii K, Gerszten R, Zheng YW, Welsh JB, Turck CW, Coughlin SR. Determinants of thrombin receptor cleavage. Receptor domains involved, specificity, and role of the P3 aspartate. *J Biol Chem* 1995; 270: 16435–16440.

24 Kahn ML, Hammes SR, Botka C, Coughlin SR. Gene and locus structure and chromosomal localization of the protease-activated receptor gene family. *J Biol Chem* 1998; 273: 23290–23296.

25 Déry O, Corvera CU, Steinhoff M, Bunnet NW. Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. *Am J Physiol Cell Physiol* 1998; 274: C1429–C1452.

26 Jenkins AL, Chinni C, De Niese MR, Blackhart B, Mackie EJ. Expression of protease-activated receptor-2 during embryonic development. *Dev Dyn* 2000; 218: 465–471.

27 Lerner DJ, Chen M, Tram T, Coughlin SR. Agonist recognition by proteinase-activated receptor 2 and thrombin receptor – importance of extracellular loop interactions for receptor function. *J Biol Chem* 1996; 271: 13943–13947.

28 Al-Ani B, Saifeddine M, Kawabata A, Hollenberg MD. Proteinase activated receptor 2: role of extracellular loop 2 for ligand-mediated activation. *Br J Pharmacol* 1999; 128: 1105–1113.

29 Bahou WF, Coller BS, Potter CL, Norton KJ, Kutok JL, Goligorsky MS. The thrombin receptor extracellular domain contains sites crucial for peptide ligand-induced activation. *J Clin Invest* 1993; 91: 1405–1413.

30 Bahou WF, Kutok JL, Wong A, Potter CL, Coller BS. Identification of a novel thrombin receptor sequence required for activation-dependent responses. *Blood* 1994; 84: 4195–4202.

31 Nanevicz T, Ishii M, Wange L et al. Mechanisms of thrombin receptor agonist specificity – Chimeric receptors and complementary mutations identify an agonist recognition site. *J Biol Chem* 1995; 270: 21619–21625.

32 Gerszten RE, Chen J, Ishii M et al. Specificity of the thrombin receptor for agonist peptide is defined by its extracellular surface. *Nature* 1994; 368: 648–651.

33 Nanevicz T, Wang L, Chen M, Ishii M, Coughlin SR. Thrombin receptor activating mutations – Alteration of an extracellular agonist recognition domain causes constitutive signaling. *J Biol Chem* 1996; 271: 702–706.

34 Böhm SK, Khitin LM, Grady EF, Aponte G, Payan DG, Bunnett NW. Mechanisms of desensitization and resensitization of proteinase-activated receptor-2. *J Biol Chem* 1996; 271: 22003–22016.

35 Chen XL, Earley K, Luo WP, Lin SH, Schilling WP. Functional expression of a human thrombin receptor in Sf9 insect cells: Evidence for an active tethered ligand. *Biochem J* 1996; 314: 603–611.

36 Brass LF. Homologous desensitization of HEL cell thrombin receptors: distinguishable roles for proteolysis and phosphorylation. *J Biol Chem* 1992; 267: 6044–6050.

37 Vouret-Craviari V, Auberger P, Pouysségur J, Van Obberghen-Schilling E. Distinct mechanisms regulate 5-HT_2 and thrombin receptor desensitization. *J Biol Chem* 1995; 270: 4813–4821.

38 Vouret-Craviari V, Grall D, Chambard J-C, Rasmussen UB, Pouysséger J, Van Obberghen-Schilling E. Post-translational and activation-dependent modifications of the G protein-coupled thrombin receptor. *J Biol Chem* 1995; 270: 8367–8372.

39 Ishii K, Chen J, Ishii M et al. Inhibition of thrombin receptor signaling by a G-protein coupled receptor kinase. Functional specificity among G-protein coupled receptor kinases. *J Biol Chem* 1994; 269: 1125–1130.

40 Iaccarino G, Rockman HA, Shotwell KF, Tomhave ED, Koch WJ. Myocardial overexpression of GRK3 in transgenic mice: evidence for in vivo selectivity of GRKs. *Am J Physiol Heart Circ Physiol* 1998; 44: H1298–H1306.

41 Hammes SR, Shapiro MJ, Coughlin SR. Shutoff and agonist-triggered internalization of protease-activated receptor 1 can be separated by mutation of putative phosphorylation sites in the cytoplasmic tail. *Biochemistry* 1999; 38: 9308–9316.

42 Shapiro MJ, Trejo J, Zeng D, Coughlin SR. Role of the thrombin receptor's cytoplasmic tail in intracellular trafficking:

Cambridge Books Online © Cambridge University Press, 2009

distinct determinants for agonist-triggered versus tonic internalization and intracellular localization. *J Biol Chem* 1996; 271: 32874–32880.
43 Ido M, Hayashi T, Gabazza EC, Suzuki K. Identification of a novel 33-kDa Ser/Thr kinase that phosphorylates the cytoplasmic tail of protease-activated receptor 1 (thrombin receptor) in human platelets. *Thromb Haemost* 2000; 83: 617–621.
44 Hein L, Ishii K, Coughlin SR, Kobilka BK. Intracellular targeting and trafficking of thrombin receptors. A novel mechanism for resensitization of a G protein-coupled receptor. *J Biol Chem* 1994; 269: 27719–27726.
45 Trejo J, Coughlin SR. The cytoplasmic tails of protease-activated receptor-1 and substance P receptor specify sorting to lysosomes *versus* recycling. *J Biol Chem* 1999; 274: 2216–2224.
46 Trejo J, Hammes SR, Coughlin SR. Termination of signaling by protease-activated receptor-1 is linked to lysosomal sorting. *Proc Natl Acad Sci USA* 1998; 95: 13698–13702.
47 Hoxie JA, Ahuja M, Belmonte E, Pizarro S, Parton RG, Brass LF. Internalization and recycling of activated thrombin receptors. *J Biol Chem* 1993; 268: 13756–13763.
48 Woolkalis MJ, DeMelfi TM, Blanchard N, Hoxie JA, Brass LF. Regulation of thrombin receptors on human umbilical vein endothelial cell. *J Biol Chem* 1995; 270: 9868–9875.
49 Déry O, Thoma MS, Wong H, Grady EF, Bunnett NW. Trafficking of proteinase-activated receptor-2 and β-arrestin-1 tagged with green fluorescent protein – β-arrestin-dependent endocytosis of a proteinase receptor. *J Biol Chem* 1999; 274: 18524–18535.
50 Schmidt VA, Nierman WC, Maglott DR et al. The human proteinase-activated receptor-3 (PAR-3) gene – Identification within a PAR gene cluster and characterization in vascular endothelial cells and platelets. *J Biol Chem* 1998; 273: 15061–15068.
51 Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J Clin Invest* 1999; 103: 879–887.
52 O'Brien PJ, Prevost N, Molino M et al. Thrombin responses in human endothelial cells – Contributions from receptors other than PAR1 include the transactivation of PAR2 by thrombin-cleaved PAR1. *J Biol Chem* 2000; 275: 13502–13509.
53 O'Brien PJ, Molino M, Kahn M, Brass LF. Protease activated receptors: theme and variations. *Oncogene* 2001; 20: 1570–1581.
54 Covic L, Gresser AL, Kuliopulos A. Biphasic kinetics of activation and signaling for PAR1 and PAR4 thrombin receptors in platelets. *Biochemistry* 2000; 39: 5458–5467.
55 Shapiro MJ, Weiss, EJ, Faruqi TR, Coughlin SR. Protease-activated receptors 1 and 4 are shut off with distinct kinetics after activation by thrombin. *J Biol Chem* 2000; 275: 25216–25221.
56 Goodwin CA, Wheeler-Jones CPD, Kakkar VV, Deadman JJ, Authi KS, Scully MF. Thrombin receptor activating peptide does not stimulate platelet procoagulant activity. *Biochem Biophys Res Commun* 1994; 202: 321–327.
57 Satoh K, Ozaki Y, Asazuma N et al. Differential mobilization of tyrosine kinases in human platelets stimulated with thrombin or thrombin receptor agonist peptide. *Biochem Biophys Res Commun* 1996; 225: 1084–1089.
58 Lasne D, Donato J, Falet H, Rendu F. Different abilities of thrombin receptor activating peptide and thrombin to induce platelet calcium rise and full release reaction. *Thromb Haemost* 1995; 74: 1323–1328.
59 Aoki T, Tomiyama Y, Honda S et al. Difference of $(Ca^{2+})_i$ movements in platelets stimulated by thrombin and TRAP: the involvement of $\alpha_{IIb}\beta_3$-mediated TXA_2 synthesis. *Thromb Haemost* 1998; 79: 1184–1190.
60 Faruqi TR, Weiss EJ, Shapiro MJ, Huang W, Coughlin SR. Structure-function analysis of protease-activated receptor 4 tethered ligand peptides – determinants of specificity and utility in assays of receptor function. *J Biol Chem* 2000; 275: 19728–19734.
61 Brass LF, Laposata M, Banga HS, Rittenhouse SE. Regulation of the phosphoinositide hydrolysis pathway in thrombin-stimulated platelets by a pertussis toxin-sensitive guanine nucleotide-binding protein. Evaluation of its contribution to platelet activation and comparisons with the adenylate cyclase inhibitory protein, G_i. *J Biol Chem* 1986; 261: 16838–16847.
62 Brass LF, Manning DR, Williams A, Woolkalis MJ, Poncz M. Receptor and G protein-mediated responses to thrombin in HEL cells. *J Biol Chem* 1991; 266: 958–965.
63 Brass LF, Vassallo RR, Jr., Belmonte E, Ahuja M, Cichowski K, Hoxie JA. Structure and function of the human platelet thrombin receptor: studies using monoclonal antibodies against a defined epitope within the receptor N-terminus. *J Biol Chem* 1992; 267: 13795–13798.
64 Norton KJ, Scarborough RM, Kutok JL, Escobedo M-A, Nannizzi L, Coller BS. Immunologic analysis of the cloned platelet thrombin receptor activation mechanism: evidence supporting receptor cleavage, release of the N-terminal peptide, and insertion of the tethered ligand into a protected environment. *Blood* 1993; 82: 2125–2136.
65 Molino M, Bainton DF, Hoxie JA, Coughlin SR, Brass LF. Thrombin receptors on human platelets: initial localization and subsequent redistribution during platelet activation. *J Biol Chem* 1997; 272: 6011–6017.
66 Harmon JT, Jamieson GA. The glycocalicin portion of platelet glycoprotein Ib expresses both high and moderate affinity binding sites for thrombin. *J Biol Chem* 1986; 261: 13224–13229.
67 Harmon JT, Jamieson GA. Thrombin binds to a high-affinity approximately 900,000-Dalton site on human platelets. *Biochemistry* 1985; 24: 58–64.
68 Chen J, Ishii M, Wang L, Ishii K, Coughlin SR. Thrombin receptor activation. Confirmation of the intramolecular tethered liganding hypothesis and discovery of an alternative intermolecular liganding mode. *J Biol Chem* 1994; 269: 16041–16045.

Cambridge Books Online © Cambridge University Press, 2009

69 Brass LF, Pizarro S, Ahuja M, Belmonte E, Stadel J, Hoxie JA. Changes in the structure and function of the human thrombin receptor during activation, internalization and recycling. *J Biol Chem* 1994; 269: 2943–2952.

70 Liu LB, Freedman J, Hornstein A, Fenton JW, II, Song YQ, Ofosu FA. Binding of thrombin to the G-protein-linked receptor, and not to glycoprotein Ib, precedes thrombin-mediated platelet activation. *J Biol Chem* 1997; 272: 1997–2004.

71 Ramachandran R, Klufas AS, Molino M, Ahuja M, Hoxie JA, Brass LF. Release of the thrombin receptor N-terminus from the surface of human platelets activated by thrombin. *Thromb Haemost* 1997; 78: 1119–1124.

72 Molino M, Blanchard N, Belmonte E et al. Proteolysis of the human platelet and endothelial cell thrombin receptor by neutrophil-derived cathepsin G. *J Biol Chem* 1995; 270: 11168–11175.

73 Cumashi A, Ansuini H, Celli N et al. Neutrophil proteases can inactivate human PAR3 and abolish the co-receptor function of PAR3 on murine platelets. *Thromb Haemost* 2001; (in press).

74 Sambrano GR, Huang W, Faruqi T, Mahrus S, Craik C, Coughlin SR. Cathepsin G activates protease-activated receptor-4 in human platelets. *J Biol Chem* 2000; 275: 6819–6823.

75 Harmon JT, Jamieson GA. Platelet activation by thrombin in the absence of the high affinity thrombin receptor. *Biochemistry* 1988; 27: 2151–2157.

76 Lopez JA, Andrews RK, Afshar-Khargan, V, Berndt MC. Bernard–Soulier syndrome. *Blood* 1998; 91: 4397–4418.

77 De Cristofaro R, De Candia E, Rutella S, Weitz JL. The Asp^{272}–Glu^{282} region of platelet glycoprotein Ibα interacts with the heparin-binding site of α-thrombin and protects the enzyme from the heparin-catalyzed inhibition by antithrombin III. *J Biol Chem* 2000; 275: 3887–3895.

78 Chow TW, Hellums DJ, Moake JL, Kroll MH. Shear stress-induced von Willebrand factor binding to platelet glycoprotein Ib initiates calcium influx associated with aggregation. *Blood* 1992; 80: 113–120.

79 Razdan K, Hellums JD, Kroll MH. Shear-stress-induced von Willebrand factor binding to platelets causes the activation of tyrosine kinase(s). *Biochem J* 1994; 302: 681–686.

80 Jackson SP, Schoenwaelder SM, Yuan Y, Rabinowitz I, Salem HH, Mitchell CA. Adhesion receptor activation of phosphatidylinositol 3-kinase. von Willebrand factor stimulates the cytoskeletal association and activation of phosphatidylinositol 3-kinase and $pp60^{c\text{-}src}$ in human platelets. *J Biol Chem* 1994; 269: 27093–27099.

81 Du X, Harris SJ, Tetaz TJ, Ginsberg MH, Berndt MC. Association of a phospholipase A_2 (14-3-3 protein) with the platelet glycoprotein Ib–IX complex. *J Biol Chem* 1994; 269: 18287–18290.

82 De Marco L, Mazzucato M, Masotti A, Fenton JW, II, Ruggeri ZM. Function of glycoprotein Ibα in platelet activation induced by α-thrombin. *J Biol Chem* 1991; 266: 23776–23783.

83 Mazzucato M, De Marco L, Masotti A, Pradella P, Bahou WF, Ruggeri ZM. Characterization of the initial α-thrombin interaction with glycoprotein Ibα in relation to platelet activation. *J Biol Chem* 1998; 273: 1880–1887.

84 De Candia E, Hall SW, Rutella S, Landolfi R, Andrews RK, De Cristofaro R. Binding of thrombin to glycoprotein Ib accelerates hydrolysis of PAR1 on intact platelets. *J Biol Chem* 2001; (in press).

85 Dörmann D, Clemetson KJ, Kehrel BE. The GPIb thrombin-binding site is essential for thrombin-induced platelet procoagulant activity. *Blood* 2000; 96: 2469–2478.

86 Ramakrishnan V, De Guzman F, Bao M, Hall SW, Laung LL, Phillips DR. A thrombin receptor function for platelet glycoprotein Ib-IX unmasked by cleavage of glycoprotein V. *Proc Natl Acad Sci USA* 2001; 98: 1823–1828.

87 McGowan EB, Ding A, Detwiler TC. Correlation of thrombin-induced glycoprotein V hydrolysis and platelet activation. *J Biol Chem* 1983; 258: 11243–11248.

88 Arnaud E, Nicaud V, Poirier O et al. Protective effect of a thrombin receptor (protease-activated receptor 1) gene polymorphism toward venous thromboembolism. *Arterioscler Thromb Vasc Biol* 2000; 20: 585–592.

89 Arnaud E, Poirier O, Aiach M, Cambien F, MONICA Members Grp. The-5061/D polymorphism of the thrombin receptor (PAR-1) gene is not related to myocardial infarction in the ECTIM study. *Thromb Haemost* 2000; 84: 722–723.

90 Compton SJ, Cairns JA, Palmer K-J, Al-Ani B, Hollenberg MD, Wall AF. A polymorphic protease-activated receptor 2 (PAR2) displaying reduced sensitivity to trypsin and differential responses to PAR agonists. *J Biol Chem* 2000; 275: 39207–39212.

91 Furman ML, Liu LB, Benoit SE, Becker RC, Barnard MR, Michelson AD. The cleaved peptide of the thrombin receptor is a strong platelet agonist. *Proc Natl Acad Sci USA* 1998; 95: 3082–3087.

92 Furman MI, Nurden P, Berndt MC et al. The cleaved peptide of PAR1 results in a redistribution of the platelet surface GPIb-IX-V complex to the surface-connected canalicular system. *Thromb Haemost* 2000; 84: 897–903.

93 Darrow AL, Fung-Leung WP, Ye RD et al. Biological consequences of thrombin receptor deficiency in mice. *Thromb Haemost* 1996; 76: 860–866.

94 Jia M, Li MX, Dunlap V, Nelson PG. The thrombin receptor mediates functional activity-dependent neuromuscular synapse reduction via protein kinase C activation in vitro. *J Neurobiol* 1999; 38: 369–381.

95 Molino M, Woolkalis MJ, Reavey-Cantwell J et al. Endothelial cell thrombin receptors and PAR-2: two protease-activated receptors located in a single cellular environment. *J Biol Chem* 1997; 272: 11133–11141.

96 Riewald M, Kravenchko V, Petrovan RJ, Brass LF, Ulevitch R, Ruf W. Gene induction by coagulation factor Xa is mediated by activation of PAR-1. *Blood* 2000; Suppl. 1: abstract.

97 Suidan HS, Bouvier J, Schaerer E, Stone SR, Monard D, Tschopp J. Granzyme A released upon stimulation on cytotoxic T lymphocytes activates the thrombin receptor on neuronal cells and astrocytes. *Proc Natl Acad Sci USA* 1994; 91: 8112–8116.

Cambridge Books Online © Cambridge University Press, 2009

98 Suidan HS, Clemetson KJ, Brown-Luedi M et al. The serine protease granzyme A does not induce platelet aggregation but inhibits responses triggered by thrombin. *Biochem J* 1996; 315: 939–945.

99 Vassallo RR, Jr., Kieber-Emmons T, Cichowski K, Brass LF. Structure/function relationships in the activation of platelet thrombin receptors by receptor-derived peptides. *J Biol Chem* 1992; 267: 6081–6085.

100 Scarborough RM, Naughton MA, Teng W et al. Tethered ligand agonist peptides. Structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. *J Biol Chem* 1992; 267: 13146–13149.

101 Hollenberg MD, Saifeddine M, Al-Ani B, Kawabata A. Proteinase-activated receptors: structural requirements for activity, receptor cross-reactivity, and receptor selectivity of receptor-activating peptides. *Can J Physiol Pharmacol* 1997; 75: 832–841.

102 Hollenberg MD, Yang S-G, Laniyonu AA, Moore GJ, Saifeddine M. Action of thrombin receptor polypeptide in gastric smooth muscle: identification of a core pentapeptide retaining full thrombin-mimetic intrinsic activity. *Mol Pharmacol* 1992; 42: 186–191.

103 Blackhart BD, Emilsson K, Nguyen D et al. Ligand cross-reactivity within the protease-activated receptor family. *J Biol Chem* 1996; 271: 16466–16471.

104 Chao BH, Kalkunte S, Maraganore JM, Stone SR. Essential groups in synthetic agonist peptides for activation of the platelet thrombin receptor. *Biochemistry* 1992; 31: 6175–6178.

105 Natarajan S, Riexinger D, Peluso M, Seiler SM. 'Tethered ligand' derived pentapeptide agonists of thrombin receptor: a study of side chain requirements for human platelet activation and GTPase stimulation. *Int J Pept Protein Res* 1995; 45: 145–151.

106 Kawabata A, Saifeddine M, Al-Ani B, Leblond L, Hollenberg MD. Evaluation of proteinase-activated receptor-1 (PAR_1) agonists and antagonists using a cultured cell receptor desensitization assay: activation of PAR_2 by PAR_1-targeted ligands. *J Pharmacol Exp Ther* 1999; 288: 358–370.

107 Hollenberg MD, Saifeddine M, Al-Ani B, Gui Y. Proteinase-activated receptor 4 (PAR_4): action of PAR_4-activating peptides in vascular and gastric tissue and lack of cross-reactivity with PAR_1 and PAR_2. *Can J Physiol Pharmacol* 1999; 77: 458–464.

108 Hollenberg MD. Protease-activated receptors: PAR4 and counting: how long is the course? *Trends Pharmacol Sci* 1999; 20: 271–273.

109 Petersen LC, Thastrup O, Hagel G et al. Exclusion of known protease activated receptors in factor VIIa-induced signal transduction. *Thromb Haemost* 2000; 83: 571–576.

110 Sorensen BB, Freskgård PO, Soegaard N, Rao LVM, Ezban M, Petersen LC. Factor VIIa-induced p44/42 mitogen-activated protein kinase activation requires the proteolytic activity of factor VIIa and is independent of the tissue factor cytoplasmic domain. *J Biol Chem* 1999; 274: 21349–21354.

111 Takeuchi T, Harris, JL, Huang W, Yan KW, Coughlin SR, Craik CS. Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. *J Biol Chem* 2000; 275: 26333–26342.

112 Renesto P, Si-Tahar M, Moniatte M et al. Specific inhibition of thrombin-induced cell activation by the neutrophil proteinases elastase, cathepsin G, and proteinase 3: evidence for distinct cleavage sites within the aminoterminal domain of the thrombin receptor. *Blood* 1997; 89: 1944–1953.

113 Sambrano GR, Weiss EJ, Zhang YW, Huang W, Coughlin SR. Role of thrombin signaling in platelets in haemotasis and thrombosis. *Nature* 2001; 413: 74–78.

Cambridge Books Online © Cambridge University Press, 2009

9

Platelet receptors: ADP

Christian Gachet and Jean-Pierre Cazenave

Institut National de la Santé et de la Recherche Médicale U.311, Etablissement Français du Sang-Alsace, Strasbourg, France

Introduction

Adenine nucleotides interact with P2 receptors, which are widely distributed in many different cell types including endothelial, smooth muscle, epithelial and blood cells, mastocytes and neurons and regulate a broad range of physiological processes. These receptors are divided into two main groups: the G protein coupled or 'metabotropic' superfamily termed P2Y and the ligand gated ion channel or 'ionotropic' superfamily termed P2X[1]. ADP was identified 40 years ago as a factor derived from red blood cells which influenced platelet adhesiveness[2,3] and induced platelet aggregation[4,5] and it rapidly became recognized as one of the most important mediators of hemostasis and thrombosis[6,7]. Evidence that ADP plays a crucial role in formation of the hemostatic plug and the pathogenesis of arterial thrombosis has accumulated during the last decades: ADP is present at near molar concentrations in platelet dense granules and is released when platelets are stimulated by other agents such as thrombin or collagen, thus reinforcing their aggregation[8,9]; inhibitors of ADP-induced platelet aggregation are effective antithrombotic drugs[10,11]; ADP removing enzymes display antithrombotic properties in animal models[12,13]; patients with defects of ADP receptors or lacking ADP in their platelet granules suffer from a bleeding diathesis[14]. Hence ADP receptors are potential pharmacological targets for antithrombotic drugs. Molecular identification of the platelet P2 receptors over the last 5 years has now made it possible to separately assign the known effects of ADP and ATP to the three cloned P2 receptors found on platelets, namely $P2Y_1$, $P2Y_{12}$ and $P2X_1$.

Effects of ADP on platelets

Signal transduction of a weak platelet agonist

Addition of exogenous ADP to washed human platelets results in shape change, reversible aggregation at physiological concentrations of ionized calcium in the external medium and finally desensitization[15,16]. Two types of aggregation response can be distinguished in vitro: primary aggregation which is reversible and does not involve the release reaction and secondary aggregation which is irreversible and associated with granule release and formation of thromboxane A_2 (TXA_2). The factors responsible for the ADP-induced secretory reaction of human platelets have been studied extensively[17] and it is now current knowledge that low concentrations of Ca^{2+} (μM range) support release from platelets stimulated with ADP in a stirred suspension, thus inducing extensive secondary aggregation[18]. Hence the ADP-induced aggregation observed in citrated platelet-rich plasma or in artificial media containing no Ca^{2+} is artefactual and does not correspond to the physiological response of platelets to ADP. The reason for the occurrence of this aggregation is presently unknown, although it might be related to the ADP receptor density (see p. 134). Transduction of the ADP signal involves a transient rise in free cytoplasmic calcium, due to mobilization of internal stores and secondary store-mediated influx and a concomitant inhibition of adenylyl cyclase[19,20]. ADP also induces an extremely rapid influx of calcium from the extracellular medium, which has been attributed to ligand gated calcium channels[21]. The demonstration that platelets from knockout mice lacking the Gαq subunit of the Gq protein do not aggregate in response to ADP[22] indicated that the phospholipase Cβ (PLCβ) pathway is necessary to raise intracellular calcium following ADP stimulation and that this is essential to platelet

aggregation. Along with the identification of the $P2Y_1$ receptor coupled to Gq, this finding closed a long debate on whether the ADP-related rise in intracellular calcium was due to inositol (1,4,5) trisphosphate (IP_3) formation or not[23]. On the other hand, the inhibition of adenylyl cyclase is a key feature of ADP-induced platelet activation but displays no direct causal relationship to aggregation[20]. Moreover, this effect of ADP is observable only when adenylyl cyclase has been prestimulated by prostaglandins or other activators. The demonstration that ADP stimulates a G protein identified as Gi_2 in human platelet membranes[24,25], provided the first evidence that at least one of the putative ADP receptors might belong to the G protein-coupled superfamily. Thus, ADP triggers at least two main biochemical pathways in platelets, a Gq/PLC mediated rise in intracellular calcium and a Gi_2 mediated inhibition of adenylyl cyclase, which suggests the involvement of two distinct G protein-coupled receptors.

ADP is a necessary cofactor for platelet activation

Although itself a weak aggregating agent, ADP is a necessary cofactor for normal activation of platelets by other agonists such as thrombin or collagen, which induce its secretion from the dense granules. In addition, low concentrations of ADP potentiate or amplify the effects of all and even weak agonists like serotonin[15], adrenaline[26] or chemokines[27,28]. A selective role of ADP as an important cofactor of phosphoinositide 3-kinase (PI 3–K) activation has been proposed on the basis of results in platelets stimulated by PAR-1 thrombin receptor activating peptides[29] and might explain the well-known stabilization by ADP of platelet aggregates induced by thrombin[30–32]. Similarly, ADP has been implicated as an important cofactor of platelet activation through cross-linking of the FcγRIIa receptor or by sera from patients with heparin-induced thrombocytopenia[33] where PI 3–K activation is pivotal. It has been shown that the Gi-coupled pathway of ADP is critically involved in synergism, with agonists triggering tyrosine kinase activities which lead to PLCγ2 activation[34]. The same phenomenon is observed when platelets are activated through the collagen receptor GPVI (Nieswandt et al., unpublished data). Thus, concomitant signalling through Gi and either Gq or tyrosine kinases, depending on the primary agonist, could be envisaged as a general mechanism by which ADP contributes to efficient platelet activation and aggregation[35].

Molecular identity of the platelet ADP receptors

The $P2Y_1$ receptor is an ADP receptor

A platelet ADP receptor was first defined on the basis of pharmacological data as the receptor responsible for ADP-induced aggregation, intracellular calcium increases and adenylyl cyclase inhibition at which ATP was believed to be a competitive antagonist. This receptor was termed $P2_T$, where T denotes thrombocytes, and was presumed to be lineage specific[36]. Initial attempts to clone such a receptor from megakaryoblastic cell line libraries were unsuccessful. However, several P2 purinoceptors were cloned in HEL cells, in particular the $P2Y_1$ receptor which was the first P2 purinoceptor to be cloned, originally from a chick brain cDNA library[37–39]. The human $P2Y_1$ receptor contains 373 amino acid residues, has the classical structure of a G-protein coupled receptor and is widely distributed in many tissues including heart, blood vessels, smooth muscle cells, connective and neural tissues, testis, prostate and ovary[1]. $P2Y_1$ mRNA was detected in megakaryoblastic cell lines (HEL, MEG-01, Dami, CHRF-288 and K562) and human platelets[40]. A milestone was passed when, after its stable expression in Jurkat cells, the human $P2Y_1$ receptor proved to be an ADP receptor at which triphosphate nucleotides were competitive antagonists. Partial agonistic responses to triphosphate nucleotides were, in fact, due to degradation of the commercial reagents into diphosphate nucleotides[40]. These studies were extended to platelets and brain capillary endothelial cells expressing the $P2Y_1$ receptor and it was shown that the agonistic effects of purified triphosphate nucleotides were due to enzymatic transformation into the diphosphate analogues by ectonucleotidases present at the surface of the cells[41]. Such results supported the hypothesis that the $P2Y_1$ receptor could be the elusive $P2_T$ receptor.

Evidence for another platelet ADP receptor

The fact that the selective $P2Y_1$ antagonists, adenosine-2′,5′-diphosphate (A2P5P), adenosine-3′,5′-diphosphate (A3P5P) and adenosine-3′,5′-phosphosulphonate (A3P5PS) inhibited ADP-induced platelet aggregation also supported a key role of the $P2Y_1$ receptor. But the fact that these antagonists had no effect on ADP-induced adenylyl cyclase inhibition led to the firm conviction that another receptor must mediate this effect of ADP on platelets. Several groups published simultaneously studies using these antagonists, all showing that the $P2Y_1$ receptor is indeed necessary although not sufficient to induce full platelet aggregation in response to ADP[42–44]. Conversely, work with inhibitors like the thieno-

Cambridge Books Online © Cambridge University Press, 2009

pyridines ticlopidine and clopidogrel or the ATP analogue 2-propylthio-β,γ-difluoromethyleneATP (AR-C66096MX) further confirmed the existence of a separate receptor responsible for the inhibition of cAMP production by ADP[44–49]. Additional evidence came from the demonstration that the $P2Y_1$ receptor was normal, at the genetic and pharmacological levels, in a patient with congenital impairment of platelet responses to ADP[50]. Finally, definite proof of the existence of a second ADP receptor distinct from $P2Y_1$ and coupled to adenylyl cyclase inhibition was provided by the generation of $P2Y_1$ receptor knockout mice, in which platelet shape change and aggregation to usual concentrations of ADP were completely abolished, whereas the ability of ADP to inhibit cAMP formation was maintained[51,52]. It is worthy of note that a first hypothesis of two distinct ADP receptors mediating platelet shape change and aggregation, on the one hand, and inhibition of adenylyl cyclase, on the other, had already been proposed by MacFarlane in 1983 on the basis of pharmacological studies[53].

Identification of the $P2Y_{12}$ receptor

This second platelet ADP receptor, suggested to be of the P2Y type since ADP is known to activate the heterotrimeric G protein Gi_2 in human platelet membranes[24], was expected to exhibit a pharmacological profile identical to that of $P2Y_1$ but with subtle differences in the selectivity of certain ligands[23]. Depending on the authors, this elusive receptor was termed P2cyc[46,51], $P2T_{AC}$[45] or $P2Y_{ADP}$[48]. It was finally cloned from human and rat platelet cDNA libraries using an expression cloning strategy in *Xenopus* oocytes designed to detect Gi-linked receptors through their coupling to cotransfected inward-rectifying K^+ channels[54]. Sequence analysis led to the identification of a new P2Y receptor designated $P2Y_{12}$. This receptor displayed the expected pharmacological characteristics of the Gi-coupled platelet ADP receptor. In particular, Chinese Hamster Ovary (CHO) cells expressing the human $P2Y_{12}$ receptor displayed ADP-mediated inhibition of cAMP formation and this effect was reversed by selective antagonists such as 2MeSAMP or C1330–7[54] and blocked by pertussis toxin, while $P2Y_1$ antagonists were ineffective. An interesting point is that this receptor contains four extracellular cysteines and the thiol reagent *p*-chloromercuriphenylsulfonic acid (*p*CMBS), known to block the ADP-induced inhibition of cAMP production in platelets[53], has been shown to inhibit ADP-induced $P2Y_{12}$ activation in *Xenopus* oocytes[54]. This effect of thiol reagents was one of the key arguments in favour of two separate ADP receptors on platelets and it is satisfying to obtain a molecular explanation. It has also been suggested that clopidogrel acts on the $P2Y_{12}$ receptor through a thiol metabolite, which could covalently modify its cysteine residues[55]. Finally, $P2Y_{12}$ appears to be mutated in a patient known to have a congenital defect of platelet aggregation to ADP related to the Gi-linked pathway (see below). The tissue distribution of this novel G protein-coupled receptor seems to be restricted to platelets and subregions of the brain including the amygdala, caudate nucleus, corpus callosum, hippocampus, substantia nigra and thalamus[54]. The $P2Y_1$ and the $P2Y_{12}$ genes are both located on chromosome 3q24–25, together with the gene coding for the UDP-glucose receptor. In terms of homology, $P2Y_{12}$ is more closely related to the latter receptor (44% identical) than to $P2Y_1$ (19% identical). The relatively poor homology with other P2 receptors probably explains why homology cloning failed to identify the Gi-coupled ADP receptor (Fig. 9.1). The same sequence has been reported for an orphan receptor SP1999, of which ADP was found to be the cognate ligand[56], having an identical tissue distribution.

Early and later concordant binding studies numbered about 400 to 1200 binding sites per platelet for 2MeSADP[53,57–60] of which approximately one-third corresponds to the $P2Y_1$ receptor and two-thirds correspond to the $P2Y_{12}$ receptor as deduced from measurements in the presence of $P2Y_{12}$ antagonists or inhibitors[44,46] and further confirmed using [^{33}P]MRS 2179 as a selective $P2Y_1$ radioligand[61].

The $P2X_1$ receptor

In addition to the G protein-coupled $P2Y_1$ and $P2Y_{12}$ receptors, platelets also express the $P2X_1$ receptor, which has been shown to be responsible for the fast calcium entry induced by ADP[62]. P2X receptors are ATP-gated ion channels which mediate rapid (within 10 ms) and selective permeability to cations. The human $P2X_1$ receptor is composed of 399 amino acids with two transmembrane domains, intracellular amino and carboxyl termini and a large extracellular loop with ten conserved cysteine residues[1]. Homomeric or heteromeric association of at least three P2X subunits is required for the formation of a pore. These receptors are broadly expressed mainly on excitable cells like smooth muscle cells, neurons and glial cells. PCR amplification demonstrated the presence of $P2X_1$ transcripts in platelets and megakaryoblastic cell lines, while the selective $P2X_1$ agonist $\alpha\beta$MeATP was found to trigger a calcium influx into fura-2 loaded human platelets[63]. Other immunological and biochemical studies have since confirmed the existence of a functional $P2X_1$ purinoceptor on blood platelets[64–66].

Cambridge Books Online © Cambridge University Press, 2009

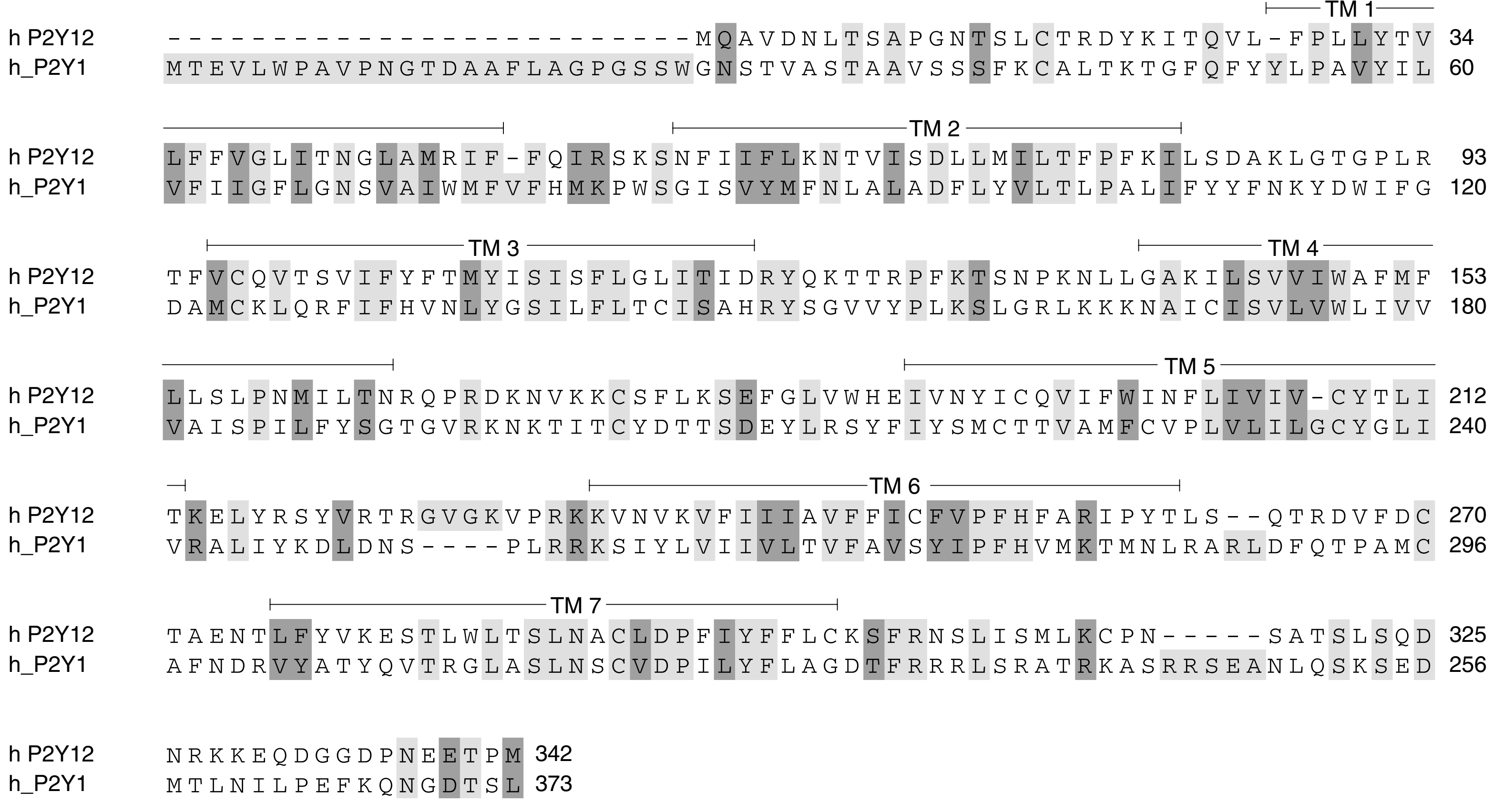


Fig. 9.1. Protein sequence alignment of the $P2Y_1$ and $P2Y_{12}$ receptors. The putative transmembrane domains are designated with bars above the sequence. Shading denotes aminoacid identity (grey) or similarity (black). The $P2Y_1$ and $P2Y_{12}$ receptors share 19% identity only.

Cambridge Books Online © Cambridge University Press, 2009

Pharmacology of the platelet ADP receptors

Agonists

The order of potency for agonists is 2MeSADP ≫ ADP = ADPαS > ADPβS at the $P2Y_1$ receptor and 2MeSADP ≫ ADP = ADPβS at the $P2Y_{12}$ receptor, at which ADPαS is an antagonist. Structure–activity studies have been extensively reviewed[16,23]. ATP has been found to have low intrinsic activity at the $P2Y_1$ receptor[67] and its action might in this case depend on the level of receptor expression. At low levels of expression, ATP would act as an antagonist and this is the case for platelets, while at higher receptor densities (transfected cells, selective tissues) it might behave as a partial agonist. The physiological significance of this behaviour is at present unclear. Concerning $P2X_1$, ATP is the 'standard' agonist and αβMeATP is a selective agonist[1]. It is not completely clear whether ADP is a true agonist of $P2X_1$ or whether the reported effects of ADP are due to contamination of the commercial sources with ATP[68]. An emerging idea is that the $P2X_1$ receptor could be activated by some diadenosine polyphosphates (Ap_nAs), and adenosine polyphospho guanosines (Ap_nGs) which are released from the dense granules of stimulated platelets[69]. These molecules have been shown to be vasoconstrictors or vasodilators depending on the local environment, and a direct effect on platelets is not excluded.

Antagonists

Selective antagonists which allow discrimination between the $P2Y_1$ and $P2Y_{12}$ receptors now exist (Table 9.1 and Fig. 9.2). A3P5PS, A2P5P and A3P5P inhibit platelet aggregation to ADP by competitively antagonizing the intracellular calcium rise induced by this agonist. These adenosine analogues are selective $P2Y_1$ receptor antagonists[70] and have no effect on adenylyl cyclase inhibition[42–44]. More efficient $P2Y_1$ antagonists such as *N*⁶-methyl-2′-deoxyadenosine-3′,5′-bisphosphate (MRS 2179) have been synthesized and shown to be antiplatelet agents[61,71,72]. However, MRS 2179 has been reported to be an antagonist of the $P2X_1$ receptor in vitro[73]. New compounds like (N)-methanocarba-*N*⁶-methyl-2-chloro-2′-deoxyadenosine-3′,5′-bisphosphate (MRS 2279) seem to be more selective and are strong antagonists of ADP-induced platelet aggregation[74,75]. Competitive antagonists of $P2Y_{12}$ also exist. Apart from 2MeSAMP and ADPαS, these are ATP analogues of the AR-C series like 2-propylthio-β,γ-difluoromethylene ATP (AR-C66096MX), 2-propylthio-β,γ-dichloromethylene ATP (AR-C67085MX) and *N*⁶-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene ATP (AR-C69931MX)[11]. They strongly inhibit ADP-induced platelet activation in vitro without interfering with shape change or calcium mobilisation. Other compounds such as C1330–7 have been reported to directly antagonize $P2Y_{12}$ but their structures have not yet been revealed[54].

Table 9.1. Antagonists and inhibitors of the platelet ADP receptors

Compounds	$P2Y_1$	$P2Y_{12}$	$P2X_1$	References
Antagonists				
A2P5P	+	−	nd	70
A3P5P	+	−	nd	70
A3P5PS	+	−	nd	70
MRS2179	+	−	+	71,73
MRS2279	+	−	−	74,75
AR-C66096MX	−	+	nd	11
AR-C67085MX	−	+	nd	11
AR-C69931MX	−	+	−	11
C1330-7	−	+	nd	54
Inhibitors				
Ticlopidine	−	+	nd	10
Clopidogrel	−	+	nd	10,55
CS-747/R-99224	−	+	nd	82,83

Ticlopidine and clopidogrel

The thienopyridine compounds ticlopidine and clopidogrel were the first $P2Y_{12}$ inhibitors to be used clinically as antithrombotic drugs[10]. In contrast to the AR-C analogues, they are inactive in vitro and have to be metabolized in the liver to acquire their antiaggregatory properties. These compounds selectively and irreversibly antagonize ADP-induced inhibition of PGE_1-activated adenylyl cyclase[76,77], binding of GTPγS to platelet membrane[78], tyrosine phosphorylation of several proteins[79] and dephosphorylation of the vasodilator-stimulated phosphoprotein VASP[80]. Conversely, they do not modify other effects of ADP on platelets like shape change, calcium movements or phosphorylation of the proteins involved in these processes. Ticlopidine and clopidogrel induce a dose-dependent reduction in the number of 2MeSADP binding sites on platelets[57–59,81]. However, under conditions where the 2MeSADP binding sites are maximally reduced (by up to 70% reduction) and ADP-induced inhibition of adenylyl cyclase is completely blocked by thienopyridine treatment, ADP can still promote platelet shape change, rise in intra-

Cambridge Books Online © Cambridge University Press, 2009

Fig. 9.2. Molecular structures of selective antagonists and inhibitors of the $P2Y_1$ and $P2Y_{12}$ receptors.

Cambridge Books Online © Cambridge University Press, 2009

cellular calcium and transient residual aggregation through activation of the $P2Y_1$ receptor, which is insensitive to thienopyridines[46,57,59]. Hence these drugs are selective $P2Y_{12}$ inhibitors. It is worthy of note that, at the maximum ex vivo effect of clopidogrel, attainable only in animals, none of the AR-C compounds have any additional effect on ADP-induced platelet aggregation, thus demonstrating that the two unrelated molecules act at the same receptor and that clopidogrel is able to fully inhibit all the receptors (Léon et al., unpublished data). The short-lived active metabolite of clopidogrel has been isolated and found to be a thiol derivative of the parent molecule. As mentioned above, it is believed to bind covalently to the $P2Y_{12}$ receptor[55]. A new antiplatelet agent (CS747) apparently identical to clopidogrel was reported recently[82] and is a thienopyridine which is totally inactive in vitro and has to be metabolized to be effective in vivo or ex vivo. One of its metabolites (R-99224) has been shown to display in vitro activity[82,83].

Congenital defects of platelet ADP receptors

Initially, two unrelated patients were described whose platelets, when exposed to ADP, changed shape normally but underwent no or only very slight, rapidly reversible aggregation and did not exhibit the normal inhibition of PGE_1-stimulated adenylyl cyclase[84,85]. Binding studies revealed a strong reduction in the number of binding sites as compared to control platelets[59,85]. There was also an abnormal pattern of protein phosphorylation upon activation by ADP or low concentrations of other agonists[86]. Thus, the clinical profile and platelet functions of these patients were the same as when thienopyridines are administered to humans or animals, suggesting them to have a $P2Y_{12}$ deficiency. This was confirmed in one patient who displays a heterozygous defect of the $P2Y_{12}$ receptor gene consisting of a dinucleotide deletion near the amino-terminal end of TM5, at amino acid 240, resulting in a 28-residue frame shift and a premature stop codon. Although they have one wild type and one mutant allele at least in the coding region, for reasons still not fully understood[54] the patient's platelets only produce RNA for the mutant receptor. Whether all the patients so far described share the same mutation will require further studies. The $P2Y_{12}$ receptor defect is inherited as an autosomal recessive trait and all the patients so far described were born from consanguineous parents. Finally, patients with secretion deficiencies but normal granule stores and TXA_2 production have been shown to have fewer ADP receptors than healthy individuals, and some of these patients are heterozygotes for the defect in $P2Y_{12}$[60,87].

A $P2Y_1$ receptor-deficient patient has been reported in abstract form[88] but has not yet been confirmed. Finally, a dominant negative mutation in the $P2X_1$ receptor consisting of a deletion of one leucine residue within a stretch of four in the second transmembrane domain (AA 351–354) has been reported in a patient with a severe bleeding disorder[89]. However, further studies will be needed to confirm these data and assess the supposedly important role of $P2X_1$ in hemostasis.

Respective roles of the $P2Y_1$, $P2Y_{12}$ and $P2X_1$ receptors in platelet functions (Fig. 9.3)

Coactivation of both $P2Y_1$ and $P2Y_{12}$ is necessary for normal ADP-induced platelet aggregation, since inhibition of either receptor is sufficient to block it. Inhibition of the $P2Y_1$ responses by selective antagonists or by gene targeting, which results in the abolition of intracellular calcium mobilization, can be bypassed by stimulating the Gq pathway with serotonin, which alone is not able to promote aggregation[43,44,51,52,90]. Similarly, inhibition of the $P2Y_{12}$ responses by clopidogrel or antagonists or its blockade in patients described above can be bypassed by adrenaline which activates Gi[46,50,90]. In this latter case, lowering cAMP is not the key signal since incubation with SQ22536, a direct inhibitor of the cyclase, of platelets from clopidogrel treated animals, did not restore ADP-induced aggregation[91]. Similarly, SQ22536 does not induce aggregation of platelets stimulated by serotonin[92]. These results show that a signalling event downstream from Gi is required for full activation of the αIIbβ3 integrin and subsequent aggregation. Nevertheless, lowering cAMP levels results in reduction of calcium removal from the cytosol, as has been evidenced in a patient with a probable $P2Y_{12}$ defect[50] and in control platelets using AR-C69931MX[93,94].

The $P2Y_1$ receptor is necessary to trigger response and is responsible for shape change and transient platelet aggregation

Adrenaline does not restore aggregation in the presence of $P2Y_1$ selective antagonists, which demonstrates that the $P2Y_1$ receptor is absolutely necessary for ADP-induced platelet aggregation[42]. This has been confirmed in $P2Y_1$ knockout mice[51,52] and likewise in studies of the well-known refractoriness of platelets to ADP[15]. The refractory state was found to be entirely due to desensitization of the $P2Y_1$ receptor, with a resultant loss of shape change and aggregation[95]. Shape change is an important event in

Cambridge Books Online © Cambridge University Press, 2009

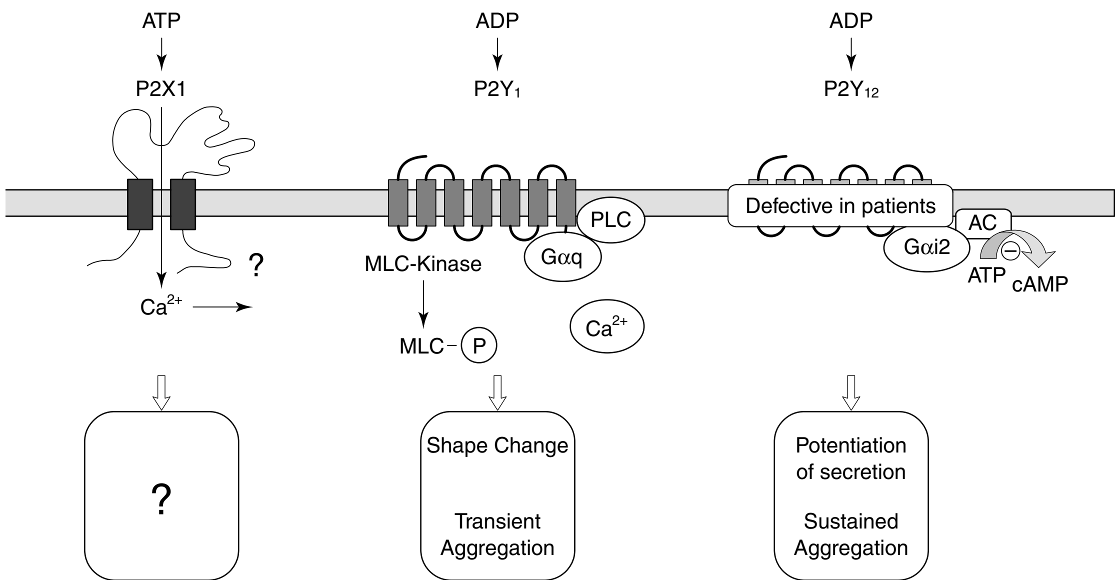


Fig. 9.3. Current view of the interplay between the platelet P2 receptors. Two G protein-coupled receptors are involved in ADP-induced platelet aggregation: the $P2Y_1$ receptor responsible for intracellular calcium mobilization, shape change and transient aggregation and the $P2Y_{12}$ receptor coupled to adenylyl cyclase inhibition, responsible for the amplification of platelet aggregation and the potentiation of platelet secretion. The latter is the molecular target of the ADP specific antiplatelet drugs thienopyridines and AR-C compounds and is defective in patients with a bleeding diathesis. Both receptors are required for normal platelet aggregation in response to ADP. Finally, a $P2X_1$ receptor is present in platelets. Responsible for rapid calcium influx, it could synergize with the $P2Y_1$ receptor. Its role in platelet activation is not yet well established.

platelet activation and is dependent on two separate biochemical pathways: a Gq-linked release of calcium from internal stores and a G12/G13-linked Rho-kinase activation[96–98]. Whether the $P2Y_1$ receptor is coupled to both pathways or only to the Gq pathway is not yet clear[98–100], but whatever its coupling, this receptor initiates platelet aggregation in response to ADP and its role is not limited to shape change. In PRP, where the concentration of fibrinogen is high (2–4 mg/ml in humans) or in washed platelet suspensions supplemented with appropriate amounts of fibrinogen, the $P2Y_1$ receptor on its own can trigger a clear, although transient, aggregation response[46] (and unpublished data). These observations better fit the aggregation profile of $P2Y_{12}$ defective patients, where a small but distinct aggregation response is obtained in PRP[84,85]. Interestingly, overexpression of $P2Y_1$ in transgenic mice results in a larger amplitude of ADP-induced platelet aggregation, even in the presence of $P2Y_{12}$ blocking agents[101]. Strikingly, ADP induces a much stronger release reaction in these murine platelets, suggesting receptor density to be the key for strong stimulation of the PLCβ and/or PLA_2 pathways.

The $P2Y_{12}$ receptor serves to amplify and complete the responses

The $P2Y_1$ receptor is nevertheless not sufficient for a full platelet response and the $P2Y_{12}$ receptor is responsible for completion and amplification of the aggregation induced by ADP and other agonists[42,45–47,49]. This receptor plays a specific role in full activation of the αIIbβ3 integrin by ADP, as has emerged from studies of patients with ADP receptor deficiencies[14]. $P2Y_{12}$ also mediates the stabilization of platelet aggregates through activation of a PI 3–K pathway downstream of Gi activation[29] and potentiation of platelet secretion[60,87]. In addition, the $P2Y_{12}$ receptor can mediate partial aggregation without shape change in platelets from $P2Y_1$ knockout mice when ADP is added at a high concentration (100 μM)[51,99], suggesting that αIIbβ3 activation can occur in the absence of $P2Y_1$ and any detectable calcium signal. The $P2Y_{12}$ receptor is also responsible for the ability of ADP to restore collagen-induced aggregation in Gαq deficient mouse platelets[99]. In general terms, it would seem that the $P2Y_{12}$ receptor is involved in all cases where ADP acts as a costimulus in the presence of low concentrations of other agonists such as thromboxane A_2, thrombin, chemokines or IgGs, while the $P2Y_1$ receptor has a specific

Cambridge Books Online © Cambridge University Press, 2009

role in early platelet activation. These respective roles of $P2Y_1$ in the early steps of aggregation and $P2Y_{12}$ in the amplification and stabilization of aggregates have been confirmed in several recent studies[93,102]. The relative contributions of the two receptors to the morphological changes induced in platelets by ADP or agonists causing ADP release have been explored in $P2Y_{12}$-deficient patients, in subjects receiving clopidogrel and in control platelets treated with selective antagonists[103,104]. It is clear from this work that $P2Y_{12}$ is involved in the constitution of stable macroaggregates through full activation of the αIIbβ3 integrin, while $P2Y_1$ is involved in the centralization of platelet granules induced by ADP and the formation of filopodia in platelets activated with low concentrations of agonists like TXA_2 or thrombin.

A role for the $P2X_1$ receptor?

One characteristic of the $P2X_1$ receptor is to become very quickly desensitized rendering difficult studies of its function in platelet activation in vitro. $P2X_1$ receptor knockout mice have been generated and display male infertility due to reduced vas deferens contraction[105], but so far no obvious defect of hemostasis. Studies of their platelet functions should help to define a role of the $P2X_1$ receptor in hemostasis and/or thrombosis. However, many investigators have, as yet, failed to find any such functional activity[45,63,106]. It has been suggested that the $P2X_1$ receptor could be implicated in shape change when care is taken to avoid desensitization by using a high concentration of apyrase at all steps of platelet handling[107]. $P2X_1$ has also been reported to synergize the ADP-induced calcium response of the $P2Y_1$ receptor, particularly under conditions where cAMP levels have been raised, which could be relevant to the in vivo situation when platelets are in contact with the vessel wall, where prostacyclin and NO are generated[94].

The platelet ADP receptors as targets for antithrombotic drugs

Since the $P2Y_1$ and $P2Y_{12}$ receptors have distinct roles in platelet activation and aggregation, they might also play separate roles in thrombosis. $P2Y_{12}$ has long been recognized as an attractive target for antithrombotic drugs. Thus, ticlopidine and clopidogrel are already commercially available, while some AR-C compounds are under evaluation in clinical trials, and both families of molecules have been shown to be efficient in animal models of thrombosis[10,11]. Ticlopidine was discovered in 1972 and already marketed in 1978, even though its precise mechanism of action was not understood. However, its selectivity as an ADP inhibitor was very quickly recognized and helped to elucidate the role of ADP in arterial thrombosis[7,108]. Clopidogrel was developed in 1986 and has been on the market since 1997[10]. This compound proved to be effective in reducing the risk of ischemic stroke, myocardial infarction or vascular death in large-scale clinical trials[10]. The AR-C compounds have proved effective in various animal models of arterial thrombosis. These compounds have already been tested in man and AR-C69931MX is in phase II clinical trials for the treatment of coronary syndromes[11]. In general terms, the pharmacological properties of the thienopyridines and AR-C analogues point to a major contribution of the $P2Y_{12}$ receptor to thrombotic states and further studies will probably lead to the discovery of new antagonists of this key target. On the other hand, the finding that $P2Y_1$-deficient mice display increased resistance to thromboembolism would suggest that this receptor could also be a target for new antiplatelet drugs[51,52]. When thromboembolism was induced by infusion of a mixture of collagen and adrenaline, $P2Y_1$ knockout mice displayed lower mortality and platelet consumption than control animals. In similar experiments, using tissue factor to promote thrombin dependent thromboembolism, $P2Y_1$-deficient mice again exhibited higher thromboresistance and significantly reduced in vivo thrombin generation[72]. Moreover, administration to mice of the $P2Y_1$-antagonist MRS 2179 resulted in prolongation of the bleeding time, inhibition of ex vivo platelet aggregation in response to ADP and resistance to thromboembolism induced by collagen and adrenaline or by tissue factor[61,72]. These results demonstrate that $P2Y_1$ represents a potential target for antithrombotic therapy.

Conclusion

ADP was identified in the 1960s as a factor influencing platelet adhesion and inducing platelet aggregation and rapidly came to be recognized as one of the most important mediators of hemostasis and thrombosis. Over the last 5 years, the platelet ADP receptors have been identified and characterized and selective P2 receptor antagonists are now under investigation as potential antithrombotic agents. Whether such $P2Y_{12}$ and $P2Y_1$ antagonists will prove useful for the antiplatelet therapy of vascular diseases should emerge in the near future.

Cambridge Books Online © Cambridge University Press, 2009

Acknowledgements

The authors would like to thank all their coworkers and colleagues from the INSERM U.311 group for their contribution to the work exposed, P. Conley (COR-Therapeutics, South San Francisco, USA) for providing the alignment of the $P2Y_1$ and $P2Y_{12}$ receptors and J. Mulvihill for correcting the English of the manuscript.

References

1 Ralevic V, Burnstock G. Receptors for purines and pyrimidines. *Pharmacol Rev* 1998; 50: 413–492.
2 Hellem A. The adhesiveness of human blood platelets in vitro. *Scand J Clin Lab Invest* 1960; 12: 1–117.
3 Gaarder A, Jonsen L, Laland S, Hellem A, Pa O. Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. *Nature* 1961; 192: 531–532.
4 Ollgard E. Macroscopic studies of platelet aggregation: nature of an aggregating factor in red blood cells and platelets. *Thromb Diath Haemorrh* 1961; 6: 86–97.
5 Born GVR. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature* 1962; 194: 27–29.
6 Born GV. Adenosine diphosphate as a mediator of platelet aggregation in vivo: an editorial view. *Circulation* 1985; 72: 741–746.
7 Maffrand JP, Bernat A, Delebassée D, Defreyn G, Cazenave JP, Gordon JL. ADP plays a key role in thrombogenesis in rats. *Thromb Haemost* 1988; 59: 225–230.
8 Reimers H. Adenine nucleotides in blood platelets. In: Longenecker G, ed. *The platelets: physiology and pharmacology*. Orlando: Academic Press; 1985: 85–98.
9 Weiss HJ. Inherited abnormalities of platelet granules and signal transduction. In Colman RW, Hirsh J, Marder VJ, Salzman EW, eds. *Hemostasis and thrombosis. Basic principles and clinical practice*. 3rd edn. Philadelphia: Lippincott J.B.; 1994: 673–684.
10 Savi P, Herbert JM. Pharmacology of ticlopidine and clopidogrel. *Haematologica* 2000; 85: 73–77.
11 Humphries RG. Pharmacology of AR-C69931MX and related compounds: from pharmacological tools to clinical trials. *Haematologica* 2000; 85: 66–72.
12 Zawilska KM, Born GV, Begent NA. Effect of ADP-utilizing enzymes on the arterial bleeding time in rats and rabbits. *Br J Haematol* 1982; 50: 317–325.
13 Marcus AJ, Broekman MJ, Drosopoulos JHF et al. Human ecto-ADPase/CD39: thromboregulation via a novel pathway. *Haematologica* 2000; 85: 53–57.
14 Cattaneo M, Gachet C. ADP receptors and clinical bleeding disorders. *Arterioscler Thromb Vasc Biol* 1999; 19: 2281–2285.
15 Macfarlane DE. Agonists and receptors: adenosine diphosphate. In Holmsen H, ed. *Platelet responses and metabolism*. Boca Raton, Florida: CRC Press; 1987: 19–36.
16 Mills DC. ADP receptors on platelets. *Thromb Haemost* 1996; 76: 835–856.
17 Mustard JF, Perry DW, Kinlough-Rathbone RL, Packham MA. Factors responsible for ADP-induced release reaction of human platelets. *Am J Physiol* 1975; 228: 1757–1765.
18 Packham MA, Bryant NL, Guccione MA, Kinlough-Rathbone RL, Mustard JF. Effect of the concentration of Ca^{2+} in the suspending medium on the responses of human and rabbit platelets to aggregating agents. *Thromb Haemost* 1989; 62: 968–976.
19 Sage SO, MacKenzie AB, Jenner S, Mahaut-Smith MP. Purinoceptor-evoked calcium signalling in human platelets. *Prostaglandins Leukot Essent Fatty Acids* 1997; 57: 435–438.
20 Haslam RJ. Interactions of the pharmacological receptors of blood platelets with adenylate cyclase. *Ser Haematol* 1973; 6: 333–350.
21 Mahaut-Smith MP, Sage SO, Rink TJ. Rapid ADP-evoked currents in human platelets recorded with the nystatin permeabilized patch technique. *J Biol Chem* 1992; 267: 3060–3065.
22 Offermanns S, Toombs CF, Hu YH, Simon MI. Defective platelet activation in G alpha(q)-deficient mice. *Nature* 1997; 389: 183–6.
23 Cusack NJ, Hourani SM. Platelet P2 receptors: from curiosity to clinical targets. *J Auton Nerv Syst* 2000; 81: 37–43.
24 Ohlmann P, Laugwitz KL, Nurnberg B et al. The human platelet ADP receptor activates Gi2 proteins. *Biochem J* 1995; 312: 775–779.
25 Gachet C, Cazenave JP, Ohlmann P, Hilf G, Wieland T, Jakobs KH. ADP receptor-induced activation of guanine-nucleotide-binding proteins in human platelet membranes. *Eur J Biochem* 1992; 207: 259–263.
26 Lanza F, Beretz A, Stierle A, Hanau D, Kubina M, Cazenave JP. Epinephrine potentiates human platelet activation but is not an aggregating agent. *Am J Physiol* 1988; 255: H1276–H1288.
27 Clemetson KJ, Clemetson JM, Proudfoot AE, Power CA, Baggiolini M, Wells TN. Functional expression of CCR1, CCR3, CCR4, and CXCR4 chemokine receptors on human platelets. *Blood* 2000; 96: 4046–4054.
28 Kowalska MA, Ratajczak MZ, Majka M et al. Stromal cell-derived factor-1 and macrophage-derived chemokine: 2 chemokines that activate platelets. *Blood* 2000; 96: 50–57.
29 Trumel C, Payrastre B, Plantavid M et al. A key role of adenosine diphosphate in the irreversible platelet aggregation induced by the PAR1-activating peptide through the late activation of phosphoinositide 3-kinase. *Blood* 1999; 94: 4156–4165.
30 Cattaneo M, Canciani MT, Lecchi A et al. Released adenosine diphosphate stabilizes thrombin-induced human platelet aggregates. *Blood* 1990; 75: 1081–1086.
31 Kinlough-Rathbone RL, Packham MA, Perry DW, Mustard JF, Cattaneo M. Lack of stability of aggregates after thrombin-induced reaggregation of thrombin-degranulated platelets. *Thromb Haemost* 1992; 67: 453–457.
32 Lau LF, Pumiglia K, Cote YP, Feinstein MB. Thrombin-receptor agonist peptides, in contrast to thrombin itself, are not full

Cambridge Books Online © Cambridge University Press, 2009

agonists for activation and signal transduction in human platelets in the absence of platelet-derived secondary mediators. *Biochem J* 1994; 303: 391–400.
33 Polgar J, Eichler P, Greinacher A, Clemetson KJ. Adenosine diphosphate (ADP) and ADP receptor play a major role in platelet activation/aggregation induced by sera from heparin-induced thrombocytopenia patients. *Blood* 1998; 91: 549–554.
34 Gratacap MP, Hérault JP, Viala C et al. FcgammaRIIA requires a Gi-dependent pathway for an efficient stimulation of phosphoinositide 3-kinase, calcium mobilization, and platelet aggregation. *Blood* 2000; 96: 3439–3446.
35 Payrastre B, Gratacap MP, Trumel C et al. ADP: an important cofactor of PI 3-kinase activation in human blood platelets. *Haematologica* 2000; 85: 32–36.
36 Gordon JL. Extracellular ATP: effects, sources and fate. *Biochem J* 1986; 233(2): 309–319.
37 Webb TE, Simon J, Krishek BJ et al. Cloning and functional expression of a brain G-protein-coupled ATP receptor. *FEBS Lett* 1993; 324: 219–225.
38 Ayyanathan K, Webbs TE, Sandhu AK, Athwal RS, Barnard EA, Kunapuli SP. Cloning and chromosomal localization of the human P2Y1 purinoceptor. *Biochem Biophys Res Commun* 1996; 218: 783–788.
39 Léon C, Vial C, Cazenave JP, Gachet C. Cloning and sequencing of a human cDNA encoding endothelial P2Y1 purinoceptor. *Gene* 1996; 171: 295–297.
40 Léon C, Hechler B, Vial C, Leray C, Cazenave JP, Gachet C. The P2Y1 receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells. *FEBS Lett* 1997; 403: 26–30.
41 Hechler B, Vigne P, Léon C, Breittmayer JP, Gachet C, Frelin C. ATP derivatives are antagonists of the P2Y1 receptor: similarities to the platelet ADP receptor. *Mol Pharmacol* 1998; 53: 727–733.
42 Hechler B, Léon C, Vial C et al. The P2Y1 receptor is necessary for adenosine 5'-diphosphate-induced platelet aggregation. *Blood* 1998; 92: 152–159.
43 Jin J, Daniel JL, Kunapuli SP. Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J Biol Chem* 1998; 273: 2030–2034.
44 Savi P, Beauverger P, Labouret C et al. Role of P2Y1 purinoceptor in ADP-induced platelet activation. *FEBS Lett* 1998; 422: 291–295.
45 Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB, Kunapuli SP. Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. *J Biol Chem* 1998; 273: 2024–2029.
46 Hechler B, Eckly A, Ohlmann P, Cazenave JP, Gachet C. The P2Y1 receptor, necessary but not sufficient to support full ADP- induced platelet aggregation, is not the target of the drug clopidogrel. *Br J Haematol* 1998; 103: 858–866.
47 Geiger J, Honig-Liedl P, Schanzenbacher P, Walter U. Ligand specificity and ticlopidine effects distinguish three human platelet ADP receptors. *Eur J Pharmacol* 1998; 351: 235–246.
48 Fagura MS, Dainty IA, McKay GD et al. P2Y1-receptors in human platelets which are pharmacologically distinct from P2Y(ADP)-receptors. *Br J Pharmacol* 1998; 124: 157–164.
49 Jantzen HM, Gousset L, Bhaskar V et al. Evidence for two distinct G-protein-coupled ADP receptors mediating platelet activation. *Thromb Haemost* 1999; 81: 111–117.
50 Léon C, Vial C, Gachet C, et al. The P2Y1 receptor is normal in a patient presenting a severe deficiency of ADP-induced platelet aggregation. *Thromb Haemost* 1999; 81: 775–781.
51 Léon C, Hechler B, Freund M et al. Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. *J Clin Invest* 1999; 104: 1731–1737.
52 Fabre JE, Nguyen M, Latour A et al. Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y1-deficient mice. *Nat Med* 1999; 5: 1199–1202.
53 Macfarlane DE, Srivastava PC, Mills DC. 2–Methylthioadenosine[beta-32P]diphosphate. An agonist and radioligand for the receptor that inhibits the accumulation of cyclic AMP in intact blood platelets. *J Clin Invest* 1983; 71: 420–428.
54 Hollopeter G, Jantzen HM, Vincent D et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* 2001; 409: 202–207.
55 Savi P, Pereillo JM, Uzabiaga MF et al. Identification and biological activity of the active metabolite of clopidogrel. *Thromb Haemost* 2000; 84: 891–896.
56 Zhang FL, Luo L, Gustafson E et al. ADP is the cognate ligand for the orphan G-protein coupled receptor SP1999. *J Biol Chem* 2000; 4: 4
57 Savi P, Laplace MC, Maffrand JP, Herbert JM. Binding of [3H]-2-methylthio ADP to rat platelets – effect of clopidogrel and ticlopidine. *J Pharmacol Exp Ther* 1994; 269: 772–777.
58 Savi P, Laplace MC, Herbert JM. Evidence for the existence of two different ADP-binding sites on rat platelets. *Thromb Res* 1994; 76: 157–169.
59 Gachet C, Cattaneo M, Ohlmann P et al. Purinoceptors on blood platelets: further pharmacological and clinical evidence to suggest the presence of two ADP receptors. *Br J Haematol* 1995; 91: 434–444.
60 Cattaneo M, Lombardi R, Zighetti ML et al. Deficiency of (33P)2MeS-ADP binding sites on platelets with secretion defect, normal granule stores and normal thromboxane A2 production. Evidence that ADP potentiates platelet secretion independently of the formation of large platelet aggregates and thromboxane A2 production. *Thromb Haemost* 1997; 77: 986–990.
61 Baurand A, Raboisson P, Freund M et al. Inhibition of platelet function by administration of MRS 2179, a $P2Y_1$ receptor antagonist. *Eur J Biochem* 2001; 412: 213–221.
62 MacKenzie AB, Mahaut-Smith MP, Sage SO. Activation of receptor-operated cation channels via P2X1 not P2T purinoceptors in human platelets. *J Biol Chem* 1996; 271: 2879–2881.
63 Vial C, Hechler B, Léon C, Cazenave JP, Gachet C. Presence of P2X1 purinoceptors in human platelets and megakaryoblastic cell lines. *Thromb Haemost* 1997; 78: 1500–1504.

Cambridge Books Online © Cambridge University Press, 2009

64 Sun B, Li J, Okahara K, Kambayashi J. P2X1 purinoceptor in human platelets. Molecular cloning and functional characterization after heterologous expression. *J Biol Chem* 1998; 273: 11544–11547.
65 Clifford EE, Parker K, Humphreys BD, Kertesy SB, Dubyak GR. The P2X1 receptor, an adenosine triphosphate-gated cation channel, is expressed in human platelets but not in human blood leukocytes. *Blood* 1998; 91: 3172–3181.
66 Scase TJ, Heath MF, Allen JM, Sage SO, Evans RJ. Identification of a P2X1 purinoceptor expressed on human platelets. *Biochem Biophys Res Commun* 1998; 242: 525–528.
67 Palmer RK, Boyer JL, Schachter JB, Nicholas RA, Harden TK. Agonist action of adenosine triphosphates at the human P2Y1 receptor. *Mol Pharmacol* 1998; 54: 1118–1123.
68 Mahaut-Smith MP, Ennion SJ, Rolf MG, Evans RJ. ADP is not an agonist at P2X(1) receptors: evidence for separate receptors stimulated by ATP and ADP on human platelets. *Br J Pharmacol* 2000; 131: 108–114.
69 Flores NA, Stavrou BM, Sheridan DJ. The effects of diadenosine polyphosphates on the cardiovascular system. *Cardiovasc Res* 1999; 42: 15–26.
70 Boyer JL, Romero-Avila T, Schachter JB, Harden TK. Identification of competitive antagonists of the P2Y1 receptor. *Mol Pharmacol* 1996; 50: 1323–1329.
71 Boyer JL, Mohanram A, Camaioni E, Jacobson KA, Harden TK. Competitive and selective antagonism of P2Y1 receptors by N6-methyl 2′- deoxyadenosine 3′,5′-bisphosphate. *Br J Pharmacol* 1998; 124: 1–3.
72 Léon C, Freund M, Ravanat C, Baurand A, Cazenave JP, Gachet C. A key role of the P2Y1 receptor in tissue factor induced thrombin dependent acute thromboembolism. Studies in P2Y1 knockout mice and mice treated with a P2Y1 antagonist. *Circulation* 2001: 103: 718–723.
73 Brown SG, King BF, Kim YC, Yang SY, Burnstock G, Jacobson KA. Activity of novel adenine nucleotide derivatives as agonists and antagonists at recombinant P2X receptors. *Drug Dev Res* 2000; 49: 253–259.
74 Boyer JL, Adams M, Jang SY, Ravi RG, Jacobson KA, Harden TK. Development of P2Y1 receptor antagonists. *Drug Dev Res* 2000; 50 (Suppl.): 21 (abstract).
75 Nandanan E, Jang SY, Moro S et al. Synthesis, biological activity, and molecular modeling of ribose-modified deoxyadenosine bisphosphate analogues as P2Y(1) receptor ligands. *J Med Chem* 2000; 43: 829–842.
76 Gachet C, Cazenave JP, Ohlmann P et al. The thienopyridine ticlopidine selectively prevents the inhibitory effects of ADP but not of adrenaline on cAMP levels raised by stimulation of the adenylate cyclase of human platelets by PGE1. *Biochem Pharmacol* 1990; 40: 2683–2687.
77 Defreyn G, Gachet C, Savi P, Driot F, Cazenave JP, Maffrand JP. Ticlopidine and clopidogrel (SR 25990C) selectively neutralize ADP inhibition of PGE1-activated platelet adenylate cyclase in rats and rabbits. *Thromb Haemost* 1991; 65: 186–190.
78 Gachet C, Savi P, Ohlmann P, Maffrand JP, Jakobs KH, Cazenave JP. ADP receptor induced activation of guanine nucleotide binding proteins in rat platelet membranes – an effect selectively blocked by the thienopyridine clopidogrel. *Thromb Haemost* 1992; 68: 79–83.
79 Savi P, Artcanuthurry V, Bornia J et al. Effect of clopidogrel treatment on ADP-induced phosphorylations in rat platelets. *Br J Haematol* 1997; 97: 185–191.
80 Geiger J, Brich J, Honig-Liedl P et al. Specific impairment of human platelet P2Y(AC) ADP receptor-mediated signalling by the antiplatelet drug clopidogrel. *Arterioscler Thromb Vasc Biol* 1999; 19: 2007–2011.
81 Mills DC, Puri R, Hu CJ et al. Clopidogrel inhibits the binding of ADP analogues to the receptor mediating inhibition of platelet adenylate cyclase. *Arterioscler Thromb* 1992; 12: 430–436.
82 Sugidachi A, Asai F, Ogawa T, Inoue T, Koike H. The in vivo pharmacological profile of CS-747, a novel antiplatelet agent with platelet ADP receptor antagonist properties. *Br J Pharmacol* 2000; 129: 1439–1446.
83 Sugidachi A, Asai F, Yoneda K et al. Antiplatelet action of R-99224, an active metabolite of a novel thienopyridine-type G(i)-linked P2T antagonist, CS-747. *Br J Pharmacol* 2001; 132: 47–54.
84 Cattaneo M, Lecchi A, Randi AM, McGregor JL, Mannucci PM. Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. *Blood* 1992; 80: 2787–2796.
85 Nurden P, Savi P, Heilmann E et al. An inherited bleeding disorder linked to a defective interaction between ADP and its receptor on platelets. Its influence on glycoprotein IIb-IIIa complex function. *J Clin Invest* 1995; 95: 1612–1622.
86 Lévy-Toledano S, Maclouf J, Rosa JP et al. Abnormal tyrosine phosphorylation linked to a defective interaction between ADP and its receptor on platelets. *Thromb Haemost* 1998; 80: 463–468.
87 Cattaneo M, Lecchi A, Lombardi R, Gachet C, Zighetti ML. Platelets from a patient heterozygous for the defect of P2(CYC) receptors for ADP have a secretion defect despite normal thromboxane A(2) production and normal granule stores: further evidence that some cases of platelet 'Primary secretion Defect' are heterozygous for a defect of P2(CYC) receptors. *Arterioscler Thromb Vasc Biol* 2000; 20: E101–E106.
88 Oury C, Lenaerts T, Peerlinck K, Vermylen J, Hoylaerts MF. Congenital deficiency of the phospholipase C coupled platelet $P2Y_1$ receptor leads to a mild bleeding disorder. *Thromb Haemost* 1999; Suppl: 20.
89 Oury C, Toth-Zsamboki E, Van Geet C et al. A natural dominant negative P2X1 receptor due to deletion of a single amino acid residue. *J Biol Chem* 2000; 275: 22611–22614.
90 Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. *Proc Natl Acad Sci USA* 1998; 95: 8070–8074.
91 Savi P, Pflieger AM, Herbert JM. cAMP is not an important messenger for ADP-induced platelet aggregation. *Blood Coagul Fibrinolysis* 1996; 7: 249–252.

Cambridge Books Online © Cambridge University Press, 2009

92 Daniel JL, Dangelmaier C, Jin J, Kim YB, Kunapuli SP. Role of intracellular signalling events in ADP-induced platelet aggregation. *Thromb Haemost* 1999; 82: 1322–1326.

93 Storey RF, Sanderson HM, White AE, May JA, Cameron KE, Heptinstall S. The central role of the P2T receptor in amplification of human platelet activation, aggregation, secretion and procoagulant activity. *Br J Haematol* 2000; 110: 925–934.

94 Sage SO, Yamoah EH, Heemskerk JW. The roles of P(2X1)and P(2T AC)receptors in ADP-evoked calcium signalling in human platelets. *Cell Calcium* 2000; 28: 119–126.

95 Baurand A, Eckly A, Bari N et al. Desensitization of the platelet aggregation response to ADP: differential down-regulation of the P2Y1 and P2cyc receptors. *Thromb Haemost* 2000; 84: 484–491.

96 Bauer M, Retzer M, Wilde JI et al. Dichotomous regulation of myosin phosphorylation and shape change by Rho-kinase and calcium in intact human platelets. *Blood* 1999; 94: 1665–1672.

97 Klages B, Brandt U, Simon MI, Schultz G, Offermanns S. Activation of G12/G13 results in shape change and Rho/Rho-kinase- mediated myosin light chain phosphorylation in mouse platelets. *J Cell Biol* 1999; 144: 745–754.

98 Paul BZ, Daniel JL, Kunapuli SP. Platelet shape change is mediated by both calcium-dependent and -independent signalling pathways. Role of p160 Rho-associated coiled-coil-containing protein kinase in platelet shape change. *J Biol Chem* 1999; 274: 28293–28300.

99 Ohlmann P, Eckly A, Freund M, Cazenave JP, Offermanns S, Gachet C. ADP induces partial platelet aggregation without shape change and potentiates collagen-induced aggregation in the absence of Galphaq. *Blood* 2000; 96: 2134–2139.

100 Wilde JI, Retzer M, Siess W, Watson SP. ADP-induced platelet shape change: an investigation of the signalling pathways involved and their dependence on the method of platelet preparation. *Platelets* 2000; 11: 286–295.

101 Hechler B, Zhang Y, Lenain N et al. Platelet hyperreactivity due to overexpression of the P2Y1 receptor in transgenic mice. *Thromb Haemost* 2001; Suppl XVIIIth Congress of the ISTH.

102 Jarvis GE, Humphries RG, Robertson MJ, Leff P. ADP can induce aggregation of human platelets via both P2Y(1) and P(2T) receptors. *Br J Pharmacol* 2000; 129: 275–282.

103 Humbert M, Nurden P, Bihour C et al. Ultrastructural studies of platelet aggregates from human subjects receiving clopidogrel and from a patient with an inherited defect of an ADP-dependent pathway of platelet activation. *Arterioscler Thromb Vasc Biol* 1996; 16: 1532–1543.

104 Eckly A, Gendrault JL, Hechler B, Cazenave JP, Gachet C. Differential involvement of the P2Y1 and P2cyc receptors in morphological changes during platelet aggregation. *Thromb Haemost* 2001; 85: in press.

105 Mulryan K, Gitterman DP, Lewis CJ et al. Reduced vas deferens contraction and male infertility in mice lacking P2X1 receptors. *Nature* 2000; 403: 86–89.

106 Takano S, Kimura J, Matsuoka I, Ono T. No requirement of P2X1 purinoceptors for platelet aggregation. *Eur J Pharmacol* 1999; 372: 305–309.

107 Rolf MG, Brearley CA, Mahaut-Smith MP. Platelet shape change evoked by selective activation of P2X1 purinoceptors with α,βmethyleneATP. *Thromb Haemost* 2001; 85: 303–308.

108 Defreyn G, Bernat A, Delebassée D, Maffrand JP. Pharmacology of ticlopidine: a review. *Semin Thromb Haemost* 1989; 15: 159–166.

Cambridge Books Online © Cambridge University Press, 2009

10

Platelet receptors: prostanoids

Roberta Vezza and Garret A. FitzGerald

Center for Experimental Therapeutics, University of Pennsylvania, Philadelphia, USA

Introduction

Prostanoids, consisting of thromboxane (Tx) and prostaglandins (PGs), are oxygenated metabolites of the polyunsaturated, essential fatty acid, arachidonic acid. Prostaglandin G/H synthase, also called cyclooxygenase (COX), catalyses the rate-limiting step in the synthesis of prostanoids. It is a bifunctional enzyme, causing biotransformation of arachidonic acid to the cyclic endoperoxides, PGG_2 to PGH_2, via sequential cyclooxygenase and peroxidase activities. Prostanoids are produced in response to diverse physical and chemical stimuli and exert their actions by binding to G protein-coupled receptors (GPCRs) which are located on the surface of the same or nearby cells. Prostanoid receptors specific for TxA_2, prostacyclin (PGI_2), PGE_2, PGD_2, and $PGF_{2\alpha}$ are termed TP, IP, EP, DP, and FP, respectively[1]. As discussed later, isoforms of some of these receptors have been identified. Each receptor binds its cognate ligand with a Kd value of 1.3–40 nM. Most prostanoids cross-react with receptors other than their own, with affinities usually more than two orders of magnitude below this range. There are also some compounds that act potently on two different receptors. For example, iloprost activates not only the IP, but also the EP1, and PGE_1 displaces the binding of ^{3}H-iloprost more potently than other IP agonists, indicating that PGE_1 binds not only to EP isoforms, but also to the IP[2]. Prostanoids play an important role in platelet physiology. The major arachidonic acid metabolite in platelets is TxA_2. TxA_2 is also synthesized by other cells, including macrophages, monocytes, and lung parenchyma[3]. In platelets, TxA_2 is produced in response to activation by conventional agonists, such as thrombin and ADP, but TxA_2 is also a platelet activator itself. PGI_2 and PGD_2, in contrast, are antiaggregatory. PGI_2 is mainly synthesized by arterial endothelial and smooth muscle cells[1]. PGD_2 is the major arachidonic acid metabolite in the brain, and is also synthesized by mast cells and spleen[4]. In the vasculature, PGD_2 can be synthesized by isomerization of PGH_2 catalysed by plasma albumin[5]. Thus, PGD_2 provides a negative feedback mechanism for regulation of platelet activation, at least in vitro. PGE_2 is synthesized mainly by endothelial cells of the microvasculature and by venous homogenates[1,6] and exerts a proaggregatory activity when combined with other stimuli, although it is not able to induce platelet aggregation by itself[7,8].

The overall homology amongst prostanoid receptors is not high, ranging approximately from 20 to 30%, although homology among receptor homologues from different species is considerably higher (79–89% between human and mouse receptors)[2]. Prostanoid receptors possess features common to other rhodopsin-type receptors, such as two cysteines in the first and second extracellular loop, consensus sequences for *N*-glycosylation of asparagine (Asn–X–Ser/Thr) in the amino-terminal extracellular portion, and serine and threonine residues in the putative cytoplasmic domain. In addition to features common to other GPCRs, particular motifs specifically conserved among the prostanoid receptors are found in several regions, such as the seventh transmembrane domain, the second extracellular loop, and the third transmembrane domain[9]. Here we review recent studies on prostanoid receptors with particular relevance to platelet function.

Thromboxane A_2 receptor (TP)

Structure

TxA_2 receptors are abundant on platelets, vascular and airway smooth muscle cells and mediate platelet aggregation and vaso- and bronchial constriction[1]. Radioligand binding studies have been performed using several ago-

Table 10.1. Summary of ^{125}I-BOP equilibrium binding data

Species	Human	Human	Human	Rat	Rat	Guinea pig
	platelets	SMC	monocytes	platelets	SMC	lung M
K_d (nM)	2.2	2.6	1.49	0.2	0.3	0.087
B_{max} (sites/cell)	1700	33 540		771	10 872	
B_{max} (fmol/mg prot)	1300	69	696			82
% specific binding	90	48		90	60	>90

Notes:
SMC: smooth muscle cells; M: membranes; prot: protein

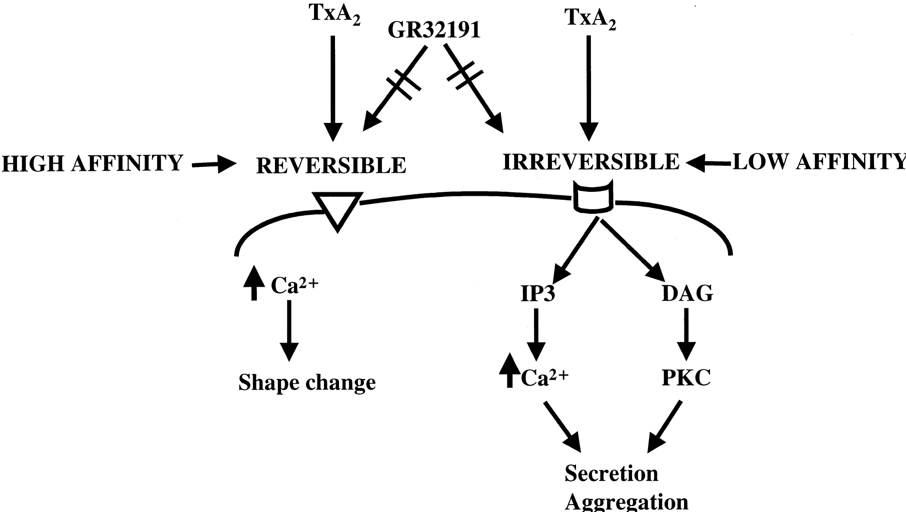


Fig. 10.1. Proposed model of the binding sites identified by GR32191 in human platelets and their effector systems. DAG: diacyglycerol; PKC: protein kinase C. Modified from Takahara et al., 1990[17].

nists and antagonists, and results using the agonist ^{125}I-BOP, a compound with high affinity for the receptor, are shown in Table 10.1[10,13].

Northern blot analysis of various mouse tissues shows presence of TP message also in immune-related organs, such as thymus and spleen[9]. PGH_2 and TxA_2 share the same pharmacological properties and activate the same receptor in platelets[14]. The platelet TxA_2 receptor is often referred to as TxA_2/PGH_2 receptor. For the sake of simplicity, we will refer to this receptor as the TP. In addition to PGH_2 and TxA_2, some isoprostanes have also been shown to act as ligands of the TP in platelets and in the vasculature in vivo[15].

Pharmacological and radioligand binding studies have identified the presence of two forms of the receptor for TxA_2 in platelets[3,16]. Platelet aggregation and secretion are mediated by receptors with low affinity for the agonist I-BOP and which bind irreversibly the antagonist GR32191 (Fig. 10.1); platelet shape change is mediated by receptors with high affinity for I-BOP which bind GR32191 in a reversible fashion (Fig. 10.1)[3,16,17].

The platelet TP was purified using ligand affinity chromatography[18]. Then, a megakaryocytic cell line (MEG01 cells) and placenta cDNA libraries were screened with a probe corresponding to the partial aminoacid sequence. The deduced aminoacid sequences of the clone obtained from placenta and of the partial clone obtained from MEG01 cells were identical[19]. Since placenta is a highly vascularized tissue, this finding suggested that the platelet receptor and the vascular receptor are identical. Thus, the platelet TP is also called 'placental isoform'. According to a more recent nomenclature, this TP isoform is called TPα.

Cambridge Books Online © Cambridge University Press, 2009

TPα has also been cloned from other megakaryocytic cell lines, such as HEL[20] and K562 cells[21] and it has been confirmed that this isoform has a deduced sequence identical to that reported for the human placental TP, consisting of 343 amino acids (Fig. 10.2). Screening of a human umbilical endothelial cell library led to the cloning of a second TP isoform, TPβ[22]. Endothelial cells express both TPα and TPβ, as detected by RT-PCR[23]. TPα and TPβ are alternative splice variants of the same gene (Fig. 10.3). The alternative splicing occurs in the carboxy terminal region after the seventh transmembrane domain. Thus, TPα and TPβ differ only in the carboxy terminal tail, which is 15 amino acids long in TPα and 79 in TPβ.

Platelets express the message for both TPα and TPβ[24] although only TPα could be detected using isoform-specific antibodies[25]. The latter study also demonstrated that the platelet TP with low ligand binding affinity that mediates platelet aggregation and secretion is the cloned isoform TPα. On the other hand, the nature of the high affinity binding site responsible for platelet shape change remains to be fully elucidated.

The TP has seven transmembrane domains (Fig. 10.4) and, similarly to other rhodopsin-type receptors, has two asparagine residues in the amino terminus (Asn^4 and Asn^{16}) that are glycosylated[19]. Glycosylation of the receptor explains the difference in molecular mass of the purified TPα (57kDa) from its calculated molecular weight of 37kDa[26]. In addition, there are two cysteine residues in the first (Cys^{105}) and in the second (Cys^{183}) extracellular loop that form a disulfide bond, important for the maintenance of the receptor structure and ligand binding. Indeed, substitution of these residues with serines eliminates TPα binding[27]. Cys^{102} is important for ligand binding and signalling properties, while Cys^{223} and Thr^{221} seem to be important in eliciting normal signalling properties, although mutation of these sites does not affect ligand binding[27]. Other mutagenesis experiments have revealed that the first transmembrane domain and, in particular, a combination of Leu^{37} with either Ala^{36} or Gly^{40}, is necessary for high affinity ligand binding[28]. Other amino acids important for optimal ligand binding are located in the seventh transmembrane domain and, curiously, substitution of Trp^{299} with Leu allows preferential agonist binding and loss of antagonist binding[29]. Other mutations seem to affect the signalling or desensitization characteristics of the TPs, rather than ligand binding properties. In particular, substitution of the hydrophobic amino acid Phe^{138} in the second intracellular loop with a more polar amino acid, such as tyrosine or aspartic acid, reduces the ability of TPs to induce activation of phospholipase C (PLC)[30]. Truncation of the last 22 amino acids of the carboxy terminal tail of the mouse TP also decreases the ability to activate PLC by roughly 50%[31].

The human TxA_2 receptor gene, mapped to chromosome band 19p13.3, is present as a single copy, spans over 15 kilobases and contains 3 exons divided by 2 introns[9,32] (Fig. 10.3). The first intron occurs in the 5′ untranslated region, and the second at the end of the sixth transmembrane domain. The alternative splicing that generates TPα and TPβ occurs at the level of the third exon. Analysis of the promoter region of the TxA_2 receptor gene revealed the presence of protein kinase C (PKC) responsive elements. Activation of PKC and induction of AP-2 or an AP-2 like DNA binding factor may be responsible for transcriptional up-regulation of TPs during megakaryocytic differentiation[33]. This finding also suggests that circulating humoral factors, such as thrombin, released during myocardial infarction, activate PKC in megakaryocytes, thus stimulating the formation and release of platelets with an increased number of TxA_2 receptors[33].

Polymorphism at the TP gene locus has been recently described in rabbits and might explain differences in vascular responses to TxA_2[34]. In humans, two single nucleotide polymorphisms in the TP gene have been described: one leads to an Arg to Leu mutation at position 60[35] and is associated with bleeding disorder (see later); the other is a synonymous substitution that has been associated with bronchial asthma in adult Japanese subjects[36].

Mice differ from humans in having only one TP isoform, that consists of 341 amino acids and, thus, is similar in length to the human TPα[37]. Mice lacking the TxA_2 receptor have been generated[38]. Bleeding time is prolonged in TP deficient mice; their platelets fail to aggregate to TP agonists and have a delayed aggregation response to collagen. Contrary to the situation in humans, activation of the mouse TP does not play a significant role in ADP-induced aggregation, since ADP-induced aggregation is normal in $TP^{-/-}$ mice. In addition, vascular responses (shock syndrome associated with systemic platelet aggregation, pulmonary thrombosis, coronary spasm, and cardiovascular collapse and sudden death) to arachidonic acid and TP agonists are absent in TP deficient mice. This study confirms that most of the known functions of TxA_2 can be accounted for by the single known *Tp* gene locus in mice.

Transgenic mice overexpressing TPβ in the vasculature have been recently generated[39]. These mice do not show abnormalities in their vasculature, and blood pressure and heart rate are normal. Mating between TPβ-overexpressing females and wild type males led to intrauterine growth retardation[39] which is commonly associated with maternal diabetes or cigarette smoking in humans, both conditions associated with increased TxA_2 biosynthesis[40,41].

Cambridge Books Online © Cambridge University Press, 2009

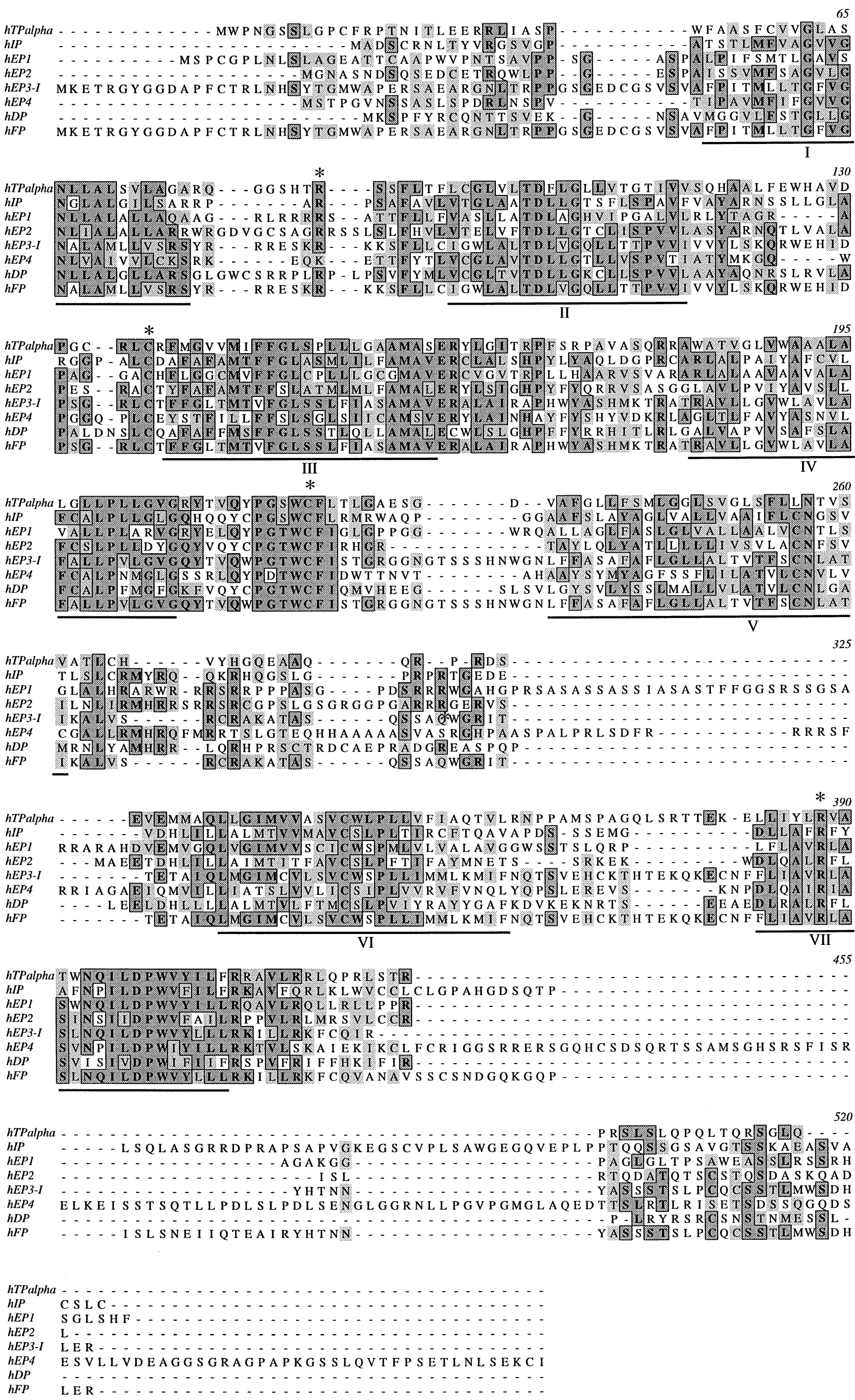


Fig. 10.2. Amino acid sequence alignment of the human prostanoid receptors. The amino acid sequences of the human TPα, IP, EP1, EP2, one of the splice variants of EP3 (EP3–I), EP4, DP, and FP are aligned to show optimal homology. The regions corresponding to the putative seven transmembrane domains are underlined. Dark grey shadows indicate amino acid identity, while light grey shadows indicate amino acid similarity. Asterisks indicate key residues, in particular: Arg in the first intracellular loop (Arg[60] in TP); Cys in the second and third extracellular loops (Cys[105] and Cys[183] in TP); Arg in the seventh transmembrane domain, conserved in all PG receptors, is proposed to be the binding site of the carboxyl group of prostanoid molecules, by analogy to the retinal binding site, Lys[296] [2]. See text for details.

Cambridge Books Online © Cambridge University Press, 2009

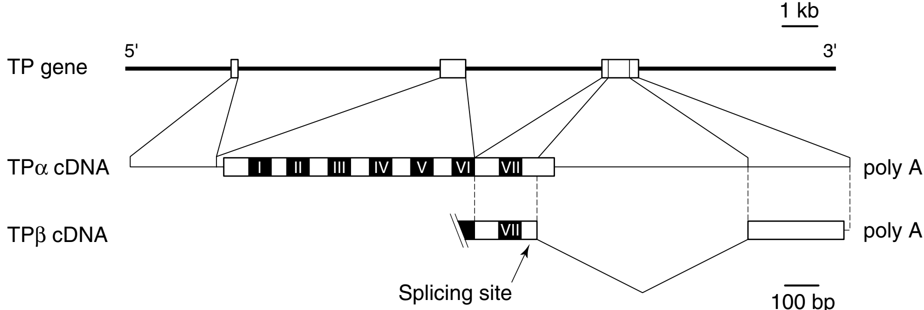


Fig. 10.3. Schematic representation of the human TP gene and TPα and TPβ cDNAs. Exons are represented by boxes. The non-coding regions are represented by horizontal lines, the regions encoding the non-membrane-spanning domains are denoted by open boxes, and the regions encoding the transmembrane domains are represented by closed boxes and are numbered. Polyadenylation sites (poly A) are also indicated. Modified from Ushikubi et al., 1995[2].

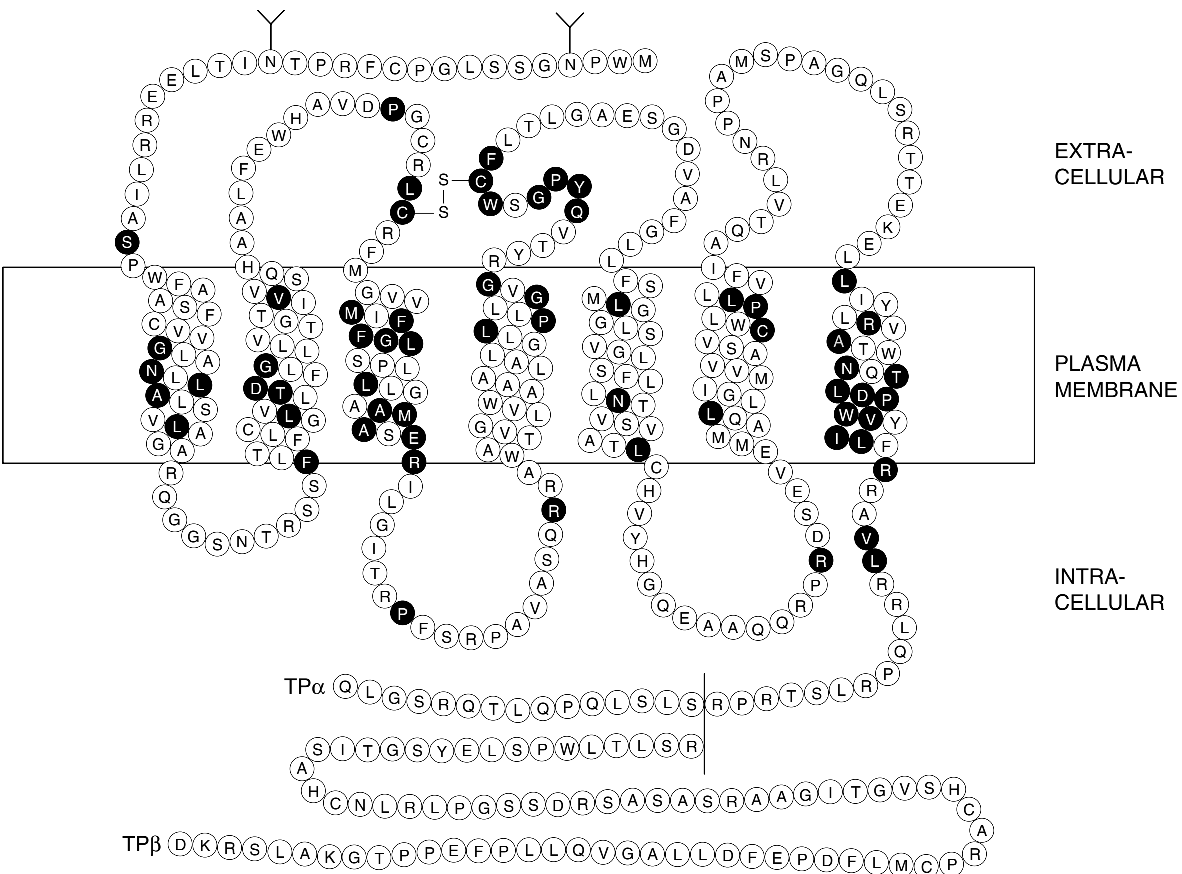


Fig. 10.4. Membrane topology model of the two human TP isoforms. Amino acid residues are indicated by single letter code. The two isoforms are different only at the level of the carboxy-terminal tail. Amino acid residues conserved by most of prostanoid receptors are shown by solid circles with white letters. N-glycosylation at Asn^{4} and Asn^{16} is indicated by the symbol Y, and putative disulfide bond between first and second extracellular loops is shown. Modified from Narumiya et al., 1999[9].

Cambridge Books Online © Cambridge University Press, 2009

Table 10.2. Signal transduction of human prostanoid receptors in overexpressing or not overexpressing systems

Type	Subtype	Isoform	G protein	Effector	Second messenger
TP		TPα	G_q, G_{11}, G_{14}, G_{16}, G_{12}, G_{13}, Gh, G_i?, G_s?	PLC AC?	↑IP ↓cAMP? ↑cAMP?
		TPβ	G_q, G_i?	PLC, AC?	↑IP ↓cAMP?
IP			G_s, G_q?	AC, PLC	↑cAMP, ↑IP
EP	EP1		Unidentified		↑Ca^{2+}
	EP2		G_s	AC	↑cAMP
	EP3	EP3-I	G_i, G_q	AC, PLC	↓cAMP, ↑IP
		EP3-II	G_i, G_s?, G_q	AC, PLC	↑cAMP? ↓cAMP ↑IP, ↑Ca^{2+}
		EP3-III	G_i, G_q	AC, PLC	↓cAMP ↑Ca^{2+}
		EP3-IV	G_q, G_i, G_s?	AC	↓cAMP, ↑cAMP? ↑Ca^{2+}
	EP4		G_s	AC	↑cAMP
DP			G_s	AC	↑cAMP

Notes:
See text for further details. IP: inositol phosphate; cAMP: cyclic AMP; AC: adenylate cyclase; ↓: decrease; ↑: increase; ? indicates either conflicting data reported by different groups, or an absence of data.

Signal transduction

Stimulation of platelets with TP agonists causes shape change, aggregation, and secretion of granule contents. These phenomena are accompanied by an increase in intracellular calcium, activation of PLC with consequent release of inositol phosphates and diacylglycerol, activation of PKC and phosphorylation of pleckstrin (also called P47), stimulation of myosin light chain kinase (MLCK) and phosphorylation of MLC, activation of phospholipase A_2 (PLA_2), and exposure of GPIIb/IIIa binding sites[3]. It is worth noting that while shape change and the increase in intracellular calcium occur independently of other platelet agonists, ADP and epinephrine are important for the occurrence of platelet aggregation following stimulation with TxA_2 analogues[42].

The effects of TxA_2 are mediated through the activation of pertussis toxin-insensitive G protein(s) (Table 10.2). Several studies have demonstrated coupling of platelet TxA_2 receptors with members of the $G\alpha_q$ family[43–45]: this may result in PLCβ activation and phosphatidyl inositol metabolism[46].

The platelet TxA_2 receptor also copurifies with an unidentified G protein of 85 kDa[44,47] that could represent the newly described G protein, $G\alpha_h$. We have shown that TPα can activate PLC in transfected cells not only through $G\alpha_q$, but also through $G\alpha_h$[48]. Arg^{60} in the first intracellular loop is important in mediating activation of PLCβ by both TPα and TPβ. It is worth noting that an arginine or another basic amino acid is present in the first intracellular loop of all of the prostanoid receptors at analogous positions[9]. Mutation of Arg^{60} impairs also adenylate cyclase stimulation by TPα, but not adenylate cylase inhibition by TPβ. These findings suggest that adenylate cyclase inhibition might be involved in some of the TxA_2-induced platelet responses, such as shape change and activation of PLA_2, which are normal in patients carrying an Arg^{60} to Leu mutation[35]. On the contrary, platelets from patients with this mutation do not aggregate in response to TxA_2 analogues, due to a defective activation of PLC[35,49].

Cambridge Books Online © Cambridge University Press, 2009

In transfected cells, TPα has been shown to mediate an increase in inositol phosphate when coexpressed not only with Gα_q, but also with other members of the Gα_q family, such as Gα_{11}, Gα_{14}, Gα_{16} and its murine counterpart, Gα_{15}[46]. The coupling of TPα with Gα_{16} is particularly interesting, since the expression of this G protein is restricted to hematopoietic cells.

Platelet TPs also couple with Gα_{12} and Gα_{13}[45]. Coupling of the TP with these G proteins may be responsible for platelet alkalinization[50], which is one of the early events in plaletet activation[51]. Another study[20] showed that the affinity status of TPα transfected into COS-7 cells can be altered by coexpression of Gα_q and Gα_{13}, but not Gα_{12}, although Gα_{12} was able to potentiate the effects of Gα_{13}. These authors hypothesize that the high and low affinity TPs responsible for platelet shape change and aggregation, respectively, and identified by radioligand studies[3,16], are not separate entities, and that the affinity status can be altered by coupling with different G proteins.

The coupling of TxA_2 receptor with Gα_i, with consequent inhibition of adenylate cyclase, is controversial. Several studies could not detect coupling of TP with Gα_i[20,43,45,52] while Ushikubi et al.[53] demonstrated reconstitution of platelet TP with Gα_{i2} in phospholipid vesicles and inhibition of iloprost-induced cyclic AMP increase in intact platelets.

Coupling of the platelet TP with Gα_s and activation of adenylate cyclase has not been demonstrated in platelets, although homologous desensitization of the TP subtype linked to PLC is accompanied by an increased sensitivity of adenylate cyclase to stimuli[54] (see later). In addition, stimulation of TPα with relatively high doses of the agonist activates adenylate cyclase in transfected CHO cells, while TPβ inhibits this enzyme[24].

Activation of adenylate cyclase by TPα, but not TPβ, has been demonstrated also in TP-null endothelial cells transfected with either TPα or TPβ. Stimulation of these cells and of wild type endothelial cells with the TP agonist, I-BOP, results in inhibition of angiogenesis, possibly mediated by increased apoptosis[23]. In addition, adenylate cyclase inhibitors and activators enhance and reduce, respectively, the apoptotic response induced by TPβ stimulation, while they do not affect TPα mediated apoptosis. Thus, no association between cyclic AMP changes and apoptosis could be established and it is suggested that stimulation of each TP isoform may inhibit angiogenesis and induce apoptosis *via* different downstream pathways[23].

Regulation

Since TxA_2 plays an amplifying role in platelet activation, one would expect that the response to this agonist would be tightly regulated. One of the mechanisms that regulates TxA_2 activity is the desensitization of the TP[3,55]. Initial uncoupling between receptor and G protein (desensitization) occurs with a $t_{1/2}$ of approximately 2 min, while prolonged exposure to the agonist results in progressive loss of binding sites from the cell surface (down-regulation). Both homologous and heterologous desensitization in platelets has been described[54]. Desensitization of the platelet TP subtype which activates PLC is accompanied by an increased sensitivity of adenylate cyclase[54], but not guanylate cyclase[56], to stimuli. Sensitization of adenylate cyclase in TP desensitized platelets might be important in disease conditions associated with increased TxA_2 production, in which the TP is desensitized[57]. In these patients, TP desensitization and enhanced sensitivity of adenylate cyclase might serve as compensatory mechanisms to limit the occurrence of thrombotic events. In addition, adenylate cyclase sensitization in TP desensitized platelets might have pharmacological implications. We have demonstrated that Tx synthase inhibitors, which redirect arachidonic acid metabolism toward the synthesis of antiaggregatory PGD_2 in platelets[5], have a more potent antiaggregatory activity in TP desensitized platelets than in control platelets[58]. This can be of relevance for the treatment of thrombotic disorders in which an in vivo desensitization of the platelet TP occurs.

In recent years, the mechanisms leading to desensitization and down-regulation of TPα and TPβ have been studied in overexpression systems.

The carboxy terminal tail of TPα and, especially, of TPβ, contains several serines and threonines that could be potential targets for phosphorylation. Phosphorylation is likely to be intrinsic to the process of desensitization, since it can lead to uncoupling with G proteins. Conflicting data have been reported concerning the role of PKC in TP desensitization. TPα and TPβ, when overexpressed in HEK293 cells, can be phosphorylated by exposure to the TP agonist, U46619, but neither PKC nor cyclic AMP-dependent protein kinase (also called protein kinase A, or PKA) seem to be responsible for this phosphorylation[59]. Nonetheless, both PKC and PKA are able to phosphorylate TPα and TPβ in vitro[60]. A study that used transfected CHO cells showed that both TPα and TPβ undergo homologous desensitization and that TPβ, but not TPα, could be desensitized by treatment of the cells with the PKC activator, PMA; activation of PKA did not have major effects on desensitization of either isoforms[61]. Thus, this study suggests that PKC-mediated

Cambridge Books Online © Cambridge University Press, 2009

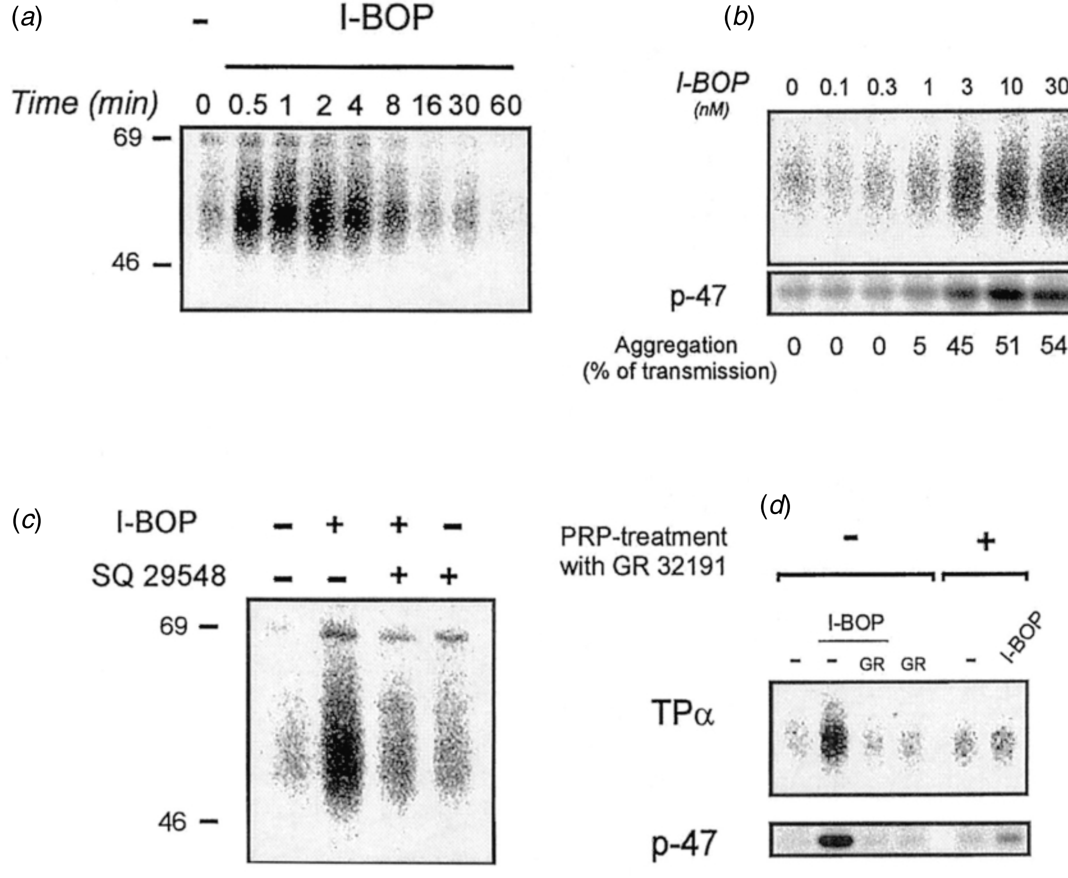


Fig. 10.5. I-BOP-induced TPα phosphorylation. [^{32}P]-labelled platelets (0.4×10^9/ml, 0.4 ml) were incubated under different conditions. Samples were immunoprecipitated using an anti TPα antibody and further subjected to SDS-PAGE. Radioactive signals were analysed using a Fuji imaging analyser. (*a*) Kinetic of TPα phosphorylation. Platelets were incubated with 10 nM I-BOP under nonstirring conditions for increasing periods of times. Results are representative of two experiments. (*b*) Dose-dependent phosphorylation of TPα was performed in the presence of I-BOP (0.1–30 nM). Corresponding aggregation is expressed as percentage of light transmission. Pleckstrin (or p-47) phosphorylation was identified after autoradiography of SDS–PAGE electrophoresis of 20 μl of total platelet lysates. These results are representative of three similar experiments. (*c*) Platelets were incubated in the presence or absence of 10 μM of SQ 29548 for 1 min at 37 °C prior to the addition of 10 nM I-BOP for 2 min. Data are representative of 3–5 experiments. (*d*) Effect of blocking low affinity binding sites of TP on TPα phosphorylation using GR 32191. Platelet-rich plasma was treated in the presence or absence of 1 μM of GR 32191 for 1 h at room temperature prior to platelet washing and labelling. Washed platelets were further incubated with 10 nM I-BOP. Platelets derived from platelet-rich plasma untreated with GR 32191 were further incubated in the presence or absence of 0.1 μM GR 32191 prior to the addition of I-BOP. P-47 phosphorylation was performed as described for (*b*). These data are representative of two similar experiments.
Reproduced from Habib et al., 1999[25].

phosphorylation plays a role in TPβ, but not TPα, desensitization. On the contrary, PKA activation following IP stimulation causes phosphorylation of TPα, but not TPβ, at Ser^{329}; this phosphorylation is accompanied by inhibition of TPα signalling[62]. Mutagenic analysis of the mouse TP revealed that PKC-mediated phosphorylation of serines located in the C-terminal tail (Ser^{321}, Ser^{322}, and Ser^{328}) is important in causing desensitization of this receptor, although the same studies suggest that PKC-induced desensitization of TP is complex and might involve mechanisms distinct from direct phosphorylation of the receptor protein[31,63]. TP phosphorylation has also been studied in human platelets, where only TPα could be detected by isoform-specific antibodies[25] (Fig. 10.5). TPα is phoshorylated in platelets following stimulation with TP agonists, arachidonic acid, collagen, thrombin, and calcium ionophore. Both homologous and

Cambridge Books Online © Cambridge University Press, 2009

heterologous phosphorylation can be blocked by PKC inhibitors[25], again suggesting the importance of PKC-mediated phosphorylation in TP regulation, at least in some experimental conditions. It is worth noting that the platelet TP is phosphorylated mainly in a PKC-dependent manner[25], while the same receptor isoform (TPα), when overexpressed in HEK293 cells, is phosphorylated independently of this enzyme[59]. This raises the possibility that heterologous expression systems may not accurately mimic the regulation of receptors in their native milieu.

Cyclic GMP-dependent protein kinase (PKG) is able to phosphorylate TPα and TPβ, independent of receptor activation. This phosphorylation, which occurs in the C terminal tail, might be important in inhibiting TP-mediated signal transduction and might explain the inhibitory activity of nitric oxide, which activates PKG, on platelet activation[64].

While phosphorylation plays a role in desensitization, down-regulation and loss of binding sites from the cell surface occurs through internalization of the receptor, following desensitization. It has been recently shown that TPα does not internalize, while TPβ does, with a plateau of ~40% after ~2–3 hours of stimulation ($t_{1/2}$~45 min). Agonist-induced internalization of TPβ might be the consequence of G protein coupled receptor kinase (GRK)-mediated phosphorylation (although this remains to be established), that might occur in the sequence between amino acids 355 and 362. Phosphorylation would increase the affinity for arrestin binding, thereby targeting the receptor for clathrin-coated pit-mediated endocytosis[65].

Another mechanism by which TPs can be regulated is at the level of gene expression. This regulation might occur in megakaryocytes, leading to production of platelets with an increased number of TPs, and also in platelets themselves. In fact, although anucleate, platelets are capable of regulated protein synthesis. It has been shown that platelets contain mRNA for Bcl-3, a member of the IκB-α family of regulatory proteins. Upon stimulation with thrombin, the message for Bcl-3 is translated and Bcl-3 protein accumulates over time. Bcl-3 synthesis in platelets is abolished by treatment with inhibitors of translation, but not with inhibitors of transcription, indicating that the Bcl-3 mRNA serves as a template for protein synthesis. Other proteins beside Bcl-3 can be synthesized upon platelet stimulation, and engagement of GPIIb/IIIa is important for initiating transcriptional events[66].

Although the mechanism is not elucidated, the PKC activator, PMA (see above and[60,67]) and testosterone[68] induce TP expression in HEL cells, a megakaryocyte-like cell line. Testosterone also increases human platelet TP density and the aggregatory response to TP agonists ex vivo[3,69]. Testosterone has been suggested as a risk factor of thrombotic disease in young athletes who abuse anabolic steroids[70].

TP antagonists have been developed and used in clinical trials for the prevention of restenosis following percutaneous transluminal coronary angioplasty (PTCA). The tested compounds did not show a significant advantage when compared to aspirin. However, the trial design included periprocedural aspirin, which suppresses the increment in PGI_2 biosynthesis induced by angioplasty[71]. Studies in mice suggest that this is of functional importance in limiting the response to injury[72] and preservation of PGI_2 biosynthesis is a potentially critical discriminant between TP antagonists and aspirin. Given the new knowledge on TPs, COX-1, and COX-2[73], the efficacy of TP antagonists in retarding atherogenesis[74], and the existence of more advanced imaging systems, it seems worthwhile to reconsider the utility of TP antagonists in cardiovascular disease.

Prostacyclin receptor (IP)

Structure

The IP is expressed by platelets, megakaryocytes, smooth muscle cells and tissues such as aorta, heart, lung, and kidney[75–77]. PGI_2 mediates inhibition of platelet aggregation and smooth muscle cell relaxation. Thus, PGI_2 antagonizes the actions of TxA_2[9] and an imbalance of these prostanoids is thought to be involved in a variety of cardiovascular disorders, such as cerebral thrombosis and ischemic heart disease[78].

The human IP has been cloned from a lung cDNA library[75,76] and a megakaryocytic leukemia cell line cDNA library[79]. It encodes a protein of 386 amino acids (Fig. 10.2) with an estimated molecular weight of 41 kDa. The IP appears to be glycosylated[80] and isoprenylated[81]. *N*-linked glycosylation at Asn[7] and especially at Asn[78] is important for proper membrane localization, ligand binding properties, and signal transduction of the IP as demonstrated by mutagenesis experiments[82].

In some studies, the cloned IP displays both a high and a low affinity binding of the ligand, [^{3}H]-iloprost[76,80], although the reason for this is presently unknown. Using ^{3}H-PGE_1 as a ligand for the IP, a low and a high affinity binding site have been detected also in human platelets[83].

Similar to the gene for the TP and DP, the gene for the human IP contains three exons separated by two introns. The first intron occurs in the 5′ untranslated region and the second at the end of the sixth transmembrane domain[84].

Cambridge Books Online © Cambridge University Press, 2009

The IP gene spans over 7 kilobases and is located on chromosome 19[84].

IP expression in megakaryocytes and in megakaryocytic cell lines is increased upon treatment with phorbol ester (which is known to induce megakaryocytic maturation), thrombopoietin (which is a potent promoter of megakaryocytopoiesis), and cytokines involved in megakaryocyte maturation or proliferation, such as interleukin-3, interleukin-6, and granulocyte–macrophage colony-stimulation factor (GM-CSF)[77]. Also proinflammatory cytokines, such as interleukin-1 and tumour necrosis factor-α upregulate IP expression, suggesting regulation of the IP gene during megakaryocyte maturation and differentiation and in inflammatory states[77].

The importance of PGI_2 as an antithrombotic agent has been demonstrated by the targeted disruption of the IP gene in mice[85]. These mice are viable, fertile, normotensive, and their heart rate, blood pressure and bleeding are normal under basal conditions, indicating that PGI_2 is not involved in the regulation of these parameters under normal circumstances. However, IP knock-out mice have an increased susceptibility to thrombosis and their response to the inflammatory stimulus, carragenan, is reduced to that observed in indomethacin-treated wild-type mice[85].

Signal transduction

Several studies have demonstrated coupling of the IP with adenylate cyclase *via* $G\alpha_s$, with a consequent increase of intracellular cyclic AMP levels[1] (Table 10.2). The increase in cyclic AMP is sufficient to inhibit platelet aggregation induced by all of the platelet agonists. Although an increase of cyclic AMP above the basal level inhibits platelet aggregation, a decrease in cyclic AMP does not favour aggregation. This has been demonstrated by using adenylate cyclase inhibitors: these compounds are not able to induce platelet aggregation, nor to potentiate aggregation induced by subthreshold concentrations of different agonists[86]. The increase in cyclic AMP leads to activation of PKA and phosphorylation of different substrates[86]. It has been recently shown that activation of platelet PKA by PGI_2 leads to phosphorylation of a G protein associated with the TxA_2 receptor, $G\alpha_{13}$. This raises the possibility that $G\alpha_{13}$ phosphorylation may be one of the mechanisms by which cyclic AMP inhibits TxA_2-induced platelet activation[87].

In addition to increasing cyclic AMP, the recombinant and the native IP also increase inositol phosphate production and intracellular calcium levels in certain cell types, but not in platelets[80,81,88,89]. This response appears to be mediated through activation of PLC via a member of the Gq family, since it is cholera and pertussis toxin insensitive[26] (Table 10.2). The EC_{50}s for cyclic AMP and inositol phosphate production in HEK293 cells stably transfected with the IP are 0.1 and 43 nM, respectively[80], although this disparity in the EC_{50} values is not evident in cells that express the receptor endogenously[88,89].

Regulation

The IP undergoes homologous desensitization followed by down-regulation. In human platelets, loss of binding sites is due to internalization of the receptor and is time and concentration dependent[90]. Platelets with internalized IPs are less responsive to the antiaggregatory effect of PGI_2[90], although the internalized receptors are not degraded and can recycle to the platelet surface within 3 hours[91]. Desensitization of the IP might be due to phosphorylation, although contrasting data have been reported in fibroblasts endogenously expressing the IP, and in transfected cells. Exposure of IP-transfected cells to a PGI_2 analogue results in a rapid, PKC-dependent, receptor phosphorylation, which is maximal within 5 minutes[80]. Ser^{328} in the carboxy-terminal tail appears to be the locus of PKC-mediated phosphorylation[92], and phosphorylation correlates with receptor desensitization. An IP mutant in which Ser^{328} is replaced by alanine does not undergo phosphorylation and is not desensitized by prior exposure of the cells to the PGI_2 analogue, iloprost[92]. It is interesting to note that the $S^{328}A$ mutant couples normally to adenylate cyclase, but not to PLC[92]. While PKC-mediated phosphorylation is important for IP desensitization in an overexpression system, this enzyme does not play a role in IP desensitization in fibroblasts naturally expressing the IP[93]. In addition, the time course of receptor desensitization in fibroblasts is much slower (1–7 hours) than in overexpressing cells and correlates with receptor internalization. Removal of the agonist after treatment of fibroblasts with iloprost for 6 hours allows recycling of the receptor to the cell surface, while more prolonged stimulation of the IP (16 hours) causes receptor degradation and *de novo* synthesis becomes a prerequisite of resensitization[93]. IP internalization following desensitization has been studied also in overexpressing cells (Fig. 10.6, see colour plate): contrary to desensitization, internalization is independent of PKC in this cellular system, and proceeds in part via a dynamin-dependent clathrin-coated vesicular endocytotic pathway[94].

Cambridge Books Online © Cambridge University Press, 2009

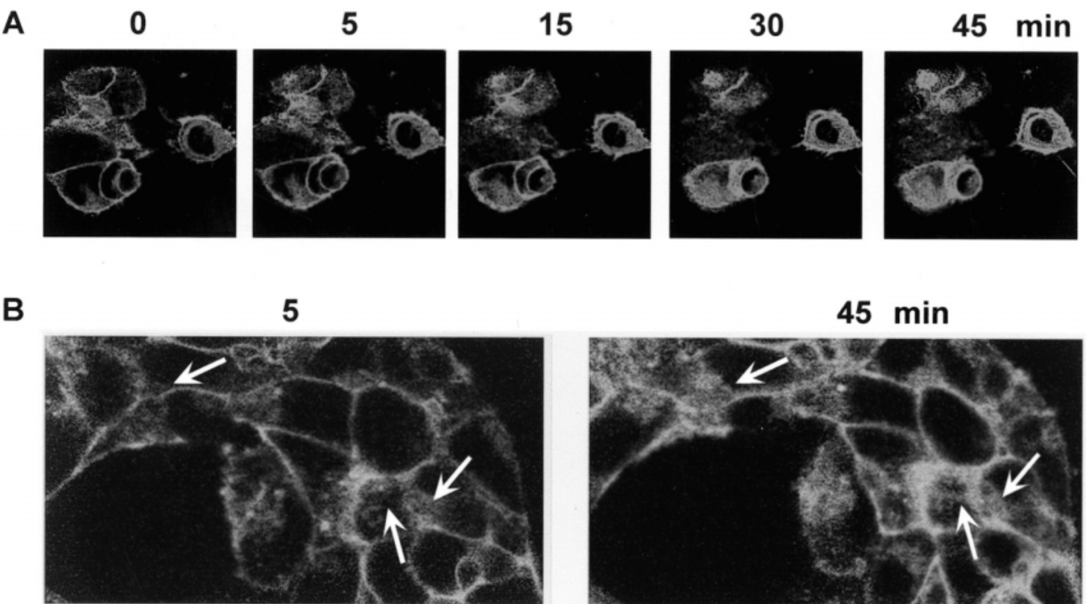


Fig. 10.6 (see also colour plate). Confocal imaging of hemagglutinine (HA) tagged human IP fused to green fluorescent protein (GFP) (HAhIP–GFP) cells in real time. (*a*) HAhIP–GFP cells were treated with 1 μM iloprost and images acquired at the indicated times. (*b*) HAhIP–GFP (green) cells were preloaded with rhodamine-conjugated transferrin (red) and images acquired at 5 and 45 min after treatment with 1 μM iloprost. Areas of co-localization (yellow) are indicated by the arrows. Data are from one experiment that was repeated with similar results. Reproduced from Smyth et al., 2000[94].

Prostaglandin E_2 receptors (EPs)

Structure

Receptors for PGE_2 are differentially expressed. Among the EPs, EP3 and EP4 are the most widely expressed, and their mRNAs have been found in almost all tissues examined. In contrast, EP1 expression is restricted to several organs, such as kidney, lung, and stomach. The EP2 is the least abundant, although its expression can be up-regulated[1,9].

The four EPs, EP1, EP2, EP3, and EP4, have been cloned from different species, including humans[9] (Fig. 10.2). The EP3 has been cloned from the erythroleukemia cell line, HEL[95], supporting earlier pharmacological studies that indicated that the platelet EP belongs to the EP3 subtype[96]. Splice variants of the EP3 have been identified in several species, including humans[97–99] (Fig. 10.7). EP3-Ia and EP3-Ib differ only in their 3' untranslated region and thus have identical open reading frames[97]. RT-PCR analysis showed that human platelets and HEL cells express several subtypes of the EP3, such as EP3-Ib, EP3-II, EP3-III, and EP3-IV, but not EP3-VI. In addition, human platelets express the EP4, but not the EP2 (both these isoforms are coupled to stimulation of adenylate cyclase), in contrast to HEL cells, that express EP2 but not EP4[100]. The expression of the EP1 was not assessed in this study. Mouse platelets express the EP3 and low levels of the EP2 and the EP4, while they do not express the EP1[101].

EP3-I	IRYHTNNYASSSTSLPCQCSSTLMWSDHLER
EP3-II	VANAVSSCSNDGQKGQPISLSNEIIQTEA
EP3-III	EEFWGN
EP3-IV	MRKRRLREQEEFWGN

Fig. 10.7. Sequence of the carboxy terminal tails of the four splice variants of the human EP3 subtype according to Kotani et al., 1995[97].

The different isoforms of the human EP3 derive from alternative splicing of the same gene[102]. The structure of the EP3 gene is very complex. It spans over more than 80 kilobases and is composed of 10 exons separated by 9 introns. Splicing occurs at the level of the carboxy terminal tail, which derives from the last portion of exon 2 or combinations of exons 3–10[102]. The EP3 isoforms are identical for the first 359 amino acids, while the C tail varies in length[97,98] (Fig. 10.2 and Fig. 10.7).

Mutagenic analysis of the second extracellular loop of the rabbit EP3 showed that this loop plays an important role in receptor–ligand interactions, but not in the signalling properties of the receptor[103]. The same study shows that mutation of Cys^{204} (which is analogous to Cys^{183} in the

Cambridge Books Online © Cambridge University Press, 2009

TP) does not cause any change in receptor binding or signalling properties: thus, a putative bridge involving Cys[204] does not seem to be important in the EP3, contrary to the situation for the TP[27].

Mice lacking the EP3 receptor have been recently generated[104]. These animals have an impaired febrile response, i.e. do not develop fever in response to endogenous (PGE_2) or exogenous (lipopolysaccharide or interleukin-1β) pyrogenes[104]. In addition, *EP3*$^{-/-}$ mice are resistant to thrombosis induced by systemic[101] or local[8] administration of arachidonic acid, although contrasting data have been reported concerning the bleeding time[8,101]. These results show that PGE_2 *via* the EP3 plays a key role in hemostasis and acute thromboembolism, at least in those models in which thrombus formation is mediated by prostanoids[101]. Thus, PGE_2 may be generated in vivo at concentrations that are in the proaggregatory range (see later). Low levels of PGE_2 appear to be necessary to augment the hemostatic effect of TxA_2, which is known to be important in hemostasis and thromboembolism in vivo[38].

Signal transduction

PGE_2 has a biphasic effect on human platelets. Low concentrations of PGE_2 (5–500 nM), although not able to induce platelet aggregation in themselves, potentiate aggregation induced by subthreshold concentrations of different agonists (collagen, ADP, thrombin, thromboxane analogues); higher and supra-physiological concentrations of PGE_2 (50–100 μM) inhibit platelet aggregation[7,8,105].

The antiaggregatory effect of high doses of PGE_2 (>50 μM) is due to increased cyclic AMP levels and is mainly the consequence of cross-activation of the IP[8]. A small contribution of the Gs-coupled EP2 and EP4 receptors in the antiaggregatory activity of PGE_2 has also been suggested[101].

The molecular mechanisms explaining the proaggregatory effect of low doses of PGE_2 are less clear. For example, it has been shown that PGE_2 induces an increase in calcium movements in human[106] and mouse platelets[101], although a calcium signal might be the consequence of cross activation of the TP[8]. We have demonstrated that proaggregatory doses of PGE_2 have a priming effect on PKC in human platelets[7]. Studies on human[107] and mouse platelets[8] have shown inhibition of adenylate cyclase by proaggregatory doses of PGE_2 (see later). Others have shown that, in human platelets, PGE_2 can either stimulate or inhibit adenylate cyclase; this may be due to the stimulation of EP4 and EP3, respectively. PGE_2 would bind to EP3 with higher affinity than to EP4 and, as a consequence, the overall effect would be an inhibition of adenylate cyclase[108]. Also the EP3 cloned from HEL and expressed in COS-1 cells has been shown to inhibit adenylate cyclase[95]. However, the results of this study are confounded by the presence of endogenous EPs in COS-1 cells that activate adenylate cyclase. In HEL cells, PGE_2 also causes an increase of intracellular calcium and, in turn, activation of phospholipase D, although it is not clear which EP is responsible for this effect[109].

Recent studies using platelets from mice deficient in EP1, EP2, EP3, or EP4 have clarified that the EP3 is the subtype responsible for the proaggregatory activity of low concentrations of PGE_2[8,101]. The proaggregatory effect of PGE_2 (100 nM) is observed in *EP1*$^{-/-}$, *EP2*$^{-/-}$ and in *EP4*$^{-/-}$ platelets, but not in *EP3*$^{-/-}$ platelets. The study of Fabre et al. suggests that PGE_2 potentiates platelet aggregation in mice by decreasing cyclic AMP levels, previously increased by antiaggregatory mediators, such as adenosine[8].

In vitro studies have shown that different EP3 isoforms differ in their ability to inhibit adenylate cyclase (Table 10.2). For example, the mouse EP3α has a marked constitutive activity, i.e. is able to activate $G\alpha_i$ and, in turn, inhibit adenylate cyclase, even in the absence of agonists, while EP3β does not display constitutive activity[110]. The level of complexity is increased even further in the bovine EP3. Four alternative splice variants of the bovine EP3 have been cloned, and termed EP3A, EP3B, EP3C, and EP3D. EP3A and EP3D, as shown for EP3 isoforms from different species, inhibit adenylate cyclase through $G\alpha_i$. Paradoxically, EP3D also activates adenylate cyclase through $G\alpha_s$ and activates PLC through a pertussis-insensitive G protein. Although EP3A also activates PLC, this most likely occurs *via* the $\beta\gamma$ subunit of $G\alpha_i$, since this effect is pertussis toxin sensitive. EP3B and EP3C only activate adenylate cyclase by coupling with $G\alpha_s$[111]. The human EP3-I inhibits adenylate cyclase and stimulates phosphoinositide turnover; EP3-II is functionally identical to the bovine EP3D, since it couples with Gi, Gs, and Gq; EP3-III inhibits adenylate cyclase; and EP3-IV is able to both inhibit and stimulate adenylate cyclase[97], although stimulation of adenylate cyclase by any of the EP3 isoforms has not been observed by another group[112]. EP3-II, EP3-III, and EP3-IV have also been shown to increase intracellular calcium in a pertussis-toxin insensitive manner[112] (Table 10.2). The human EP3-III and EP3-IV show constitutive activity toward inhibition of adenylate cyclase, while EP3-I and EP3-II inhibit adenylate cylase only in an agonist-dependent manner[113].

Regulation

Desensitization and down-regulation of EP3 isoforms have been studied following overexpression of the mouse EP3α

Cambridge Books Online © Cambridge University Press, 2009

and EP3β in CHO cells. Exposure of EP3α overexpressing cells to PGE_2 causes desensitization and downregulation of the receptor. Long-term exposure of the cells to PGE_2 causes internalization of EP3α and loss of the receptor from the cell surface: 50% of the binding sites are lost after 5 h, while up to 80% of the binding sites are lost within 24 h. In sharp contrast, EP3β does not undergo desensitization nor down-regulation. The difference between the ability of EP3α and EP3β to undergo regulation might be due to the different amino acid composition and hydrophobicity of their carboxy terminal tails. The C tail of EP3β may be incorporated into the membrane, due to its high hydrophobicity, while that of EP3α is likely to be cytoplasmic, more accessible to receptor kinases and, as a consequence, phosphorylation and desensitization. In addition, the higher number of serines and threonines and the presence of one tyrosine residue in the C tail of EP3α, but not of EP3β, can contribute to the differential regulation of these two EP3 isoforms[114]. A differential pattern of desensitization has been observed also for the human EP3 isoforms. EP3-II down-regulation occurs within 3 hours and persists up to 12 hours, while isoforms EP3-III and EP3-IV undergo a more rapid down-regulation (within ½ hour) which reverts to control level by 3 hours[112].

Prostaglandin D_2 receptor (DP)

Structure

The DP is, among the prostanoid receptors, the most restricted in its expression. It is present on human, but not mouse platelets[101], neutrophils, non-chromaffin cells from adrenal medulla, smooth muscle cells from several tissues, and nervous tissue. The DP is usually expressed in association with other prostanoid receptors and PGD_2 itself behaves as an agonist toward the TP and the FP. Mouse[115] and human[116] DP have been recently cloned. Northern blot analysis has shown that DP expression is restricted to few tissues. In mice, the DP is expressed in ileum and very weakly in lung, stomach and uterus[115], while in humans the DP is expressed in retina and in the small intestine[116]. The human DP consists of 359 amino acids (Fig. 10.2) with a calculated molecular mass of 40 kDa. There are three potential glycosylation sites in the *N*-terminus and in the first and third extracellular loops, potential PKC phosphorylation sites in the first and second cytoplasmic loops, and other potential phosphorylation sites in the C-terminal tail[115,116]. The DP gene contains an open reading frame consisting of two exons separated by an intron at a position homologous to that of the TP[115,116].

A DP-deficient mouse has been recently described[117]. Although no platelet abnormalities have been reported, it is interesting to note here that $DP^{-/-}$ mice are less susceptible to allergic asthma. Ovalbumin challenge in wild-type mice results in increased acetylcholine sensitivity, lymphocyte accumulation and increased concentration of T_H2 cytokines in the lungs. These responses were significantly lower in $DP^{-/-}$ mice, indicating that DP deficiency is manifested locally at the site of challenge[117].

Signal transduction

PGD_2 inhibits platelet aggregation in a species-specific manner, since human platelets, but not platelets from most laboratory animals, respond to PGD_2. PGD_2 also causes vasodilation and is important in the central nervous system, where it has been shown to induce sleep, hyperalgesia, regulation of body temperature and odor responses[1,9,118]. PGD_2 is abundantly released by mast cells, and plays a role during immunologic reactions.

The DP activates adenylate cyclase through Gs (Table 10.2), and the consequent increase of cyclic AMP is responsible for inhibition of platelet aggregation[1]. Although the cloned DP expressed in HEK293 cells can induce an increase of intracellular calcium upon stimulation, this response is not due to PLC activation, since no increase of inositol phosphates could be detected[115,116]. Rather, it appears that the calcium increase might be a consequence of the increased cyclic AMP, although the mechanism is presently unknown[116].

Regulation

DP regulation has not been studied in great detail. Although one could imagine that this receptor, like other GPCRs, undergoes desensitization and down-regulation, and that phosphorylation might play a role in receptor regulation, these events have not been studied in DP-expressing cells. There is one report that indirectly demonstrates regulation of the DP by phosphorylation and dephosphorylation. Binding of $^3H\text{-}PGD_2$ to membranes of the mastocytoma cell line, P-815, is increased upon treatment with phosphatase inhibitors (NaF and molybdate) and decreased upon treatment with phosphatases. In addition, the binding activity could be partially restored by treatment with molybdate[119], suggesting that reversible phosphorylation of the receptor might occur as a result of the action of unidentified protein kinase(s) and phosphatase(s) present in the membranes.

Cambridge Books Online © Cambridge University Press, 2009

Summary

Prostaglandins play important roles in platelet pathophysiology. TxA_2 is a potent platelet activating agent, while PGI_2 and PGD_2 are antiaggregatory. PGE_2 does not aggregate platelets by itself, but potentiates aggregation induced by other stimuli, while $PGF_{2\alpha}$ does not have any effect on platelet function, at least at physiological concentrations. It seems likely that the diverse actions of the different prostanoids are important in platelet homeostasis. Receptors specific for each prostanoid have been cloned and splice variants for the TP and the EP have been found. The functions of these receptors have been highlighted by the generation of knock out and transgenic mice.

Understanding prostanoid receptor function and regulation might result in improvement of therapeutic strategies to regulate platelet activation.

Acknowledgements

The assistance of Mrs Mary Thomas and Ellina Cheskis with the preparation of the figures is gratefully acknowledged.

Supported by grants from the National Institute of Health (HL57847 and HL54500 to G.A.F.) and the American Heart Association Southeastern Pennsylvania Affiliate (to R.V.). Dr FitzGerald is the Robinette Foundation Professor of Cardiovascular Medicine.

References

1 Coleman RA, Smith WL, Narumiya S. VIII International Union of Pharmacology. Classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol Rev* 1994; 46: 205–229.
2 Ushikubi F, Hirata M, Narumiya S. Molecular biology of prostanoid receptors; an overview. *J Lipid Mediat Cell Signalling* 1995; 12: 343–359.
3 Halushka PV, Allan CJ, Davis-Bruno KL. Thromboxane A_2 receptors. *J Lipid Mediat Cell Signaling* 1995; 12: 361–378.
4 Funk CD. Molecular biology in the eicosanoid field. *Prog Nucl Acid Res Mol Biol*, 1993; 45: 67–98.
5 Gresele P, Deckmyn H, Huybrechts E, Vermylen J. Serum albumin enhances the impairment of platelet aggregation with thromboxane synthase inhibition by increasing the formation of prostaglandin D_2. *Biochem Pharmacol* 1984; 33: 2083–2088.
6 Charo IF, Shak S, Kerasek MA, Davison PM, Goldstein IM. Prostaglandin I_2 is not a major metabolite of arachidonic acid metabolism in cultured endothelial cells from human foreskin microvessels. *J Clin Invest* 1984; 74: 914–919.
7 Vezza R, Roberti R, Nenci GG, Gresele P. Prostaglandin E_2 potentiates platelet aggregation by priming protein kinase C. *Blood* 1993; 82: 2704–2713.
8 Fabre JE, Nguyen MT, Athirakul K et al. Activation of the murine EP3 receptor for PGE_2 inhibits cAMP production and promotes platelet aggregation. *J Clin Invest* 2001; 107(5): 603–610.
9 Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structure, properties, and functions. *Physiol Rev* 1999; 79: 1193–1226.
10 Morinelli TA, Naka M, Masuda A et al. Characterization of thromboxane A_2/prostaglandin H_2 receptors of various tissues using a new radiodinated thromboxane A_2/prostaglandin H_2 mimetic, I-BOP. *Adv Prostaglandin Thromboxane Leukotriene Res* 1990; 21: 331–337.
11 Morinelli TA, Mais DE, Oatis JE, Crumbley AJ, Halushka PV. Characterization of thromboxane A_2/ prostaglandin H_2 receptors in human vascular smooth muscle cells. *Life Sci* 1990; 46: 1765–1772.
12 Mais DE, Saussy DL, Magee DE, Williams CM. Binding of a radiodinated agonist to thromboxane A_2/prostaglandin H_2 (TxA_2/PGH_2) receptors in guinea pig lung membranes. *Adv Prostaglandins Thromboxane Leukotriene Res* 1990; 21: 347–350.
13 Allan CJ, Halushka PV. Characterization of human peripheral blood monocyte thromboxane A_2 receptors. *J Pharmacol Exp Ther* 1994; 270: 446–452.
14 Vezza R, Mezzasoma A, Venditti G, Gresele P. Prostaglandin endoperoxides mediate calcium increase and aggregation in human platelets by acting on the same receptor isoform(s) as thromboxane A_2. *Thromb Haemost* 2002; 87 (in press).
15 Audoly LP, Rocca B, Fabre JE et al. Cardiovascular responses to the isoprostanes $iPF_{2\alpha}$-III and iPE_2-III are mediated *via* the thromboxane A_2 receptor in vivo. *Circulation* 2000; 101: 2833–2840.
16 Morinelli TA, Halushka PV. Thromboxane A_2/Prostaglandin H_2 receptors. Characterization and antagonism. *Trends Cardiovasc Med* 1991; 1: 157–161.
17 Takahara K, Murray R, FitzGerald GA, Fitzgerald DJ. The response to thromboxane A_2 analogues in human platelets. Discrimination of two binding sites linked to distinct effector systems. *J Biol Chem* 1990; 265: 6836–6844.
18 Ushikubi F, Nakajima M, Hirata M, Okuma M, Fujiwara M, Narumiya S. Purification of the thromboxane A_2/prostaglandin H_2 receptor from human blood platelets. *J Biol Chem* 1989; 264: 16496–16501.
19 Hirata M, Hayashi Y, Ushikubi F et al. Cloning and expression of cDNA for a human thromboxane A_2 receptor. *Nature* 1991; 349: 617–620.
20 Allan CJ, Higashiura K, Martin M et al. Characterization of the cloned HEL cell thromboxane A_2 receptor: evidence that the affinity state can be altered by $G\alpha_{13}$ and $G\alpha_q$. *J Pharmacol Exp Ther* 1996; 277: 1132–1139.

Cambridge Books Online © Cambridge University Press, 2009

21 D'Angelo DD, Davis MG, Ali S, Dorn GW II. Cloning and pharmacologic characterization of a thromboxane A_2 receptor from K562 (human chronic myelogenous leukemia) cells. *J Pharmacol Exp Ther* 1994; 271: 1034–1041.

22 Raychowdhury MK, Yukawa M, Collins LJ, McGrail SH, Kent KC, Ware JA. Alternative splicing produces a divergent cytoplasmic tail in the human endothelial thromboxane A_2 receptor. *J Biol Chem* 1994; 269: 19256–19261.

23 Gao Y, Yokota R, Tang S, Ashton AW, Ware JA. Reversal of angiogenesis in vitro, induction of apoptosis, and inhibition of Akt phosphorylation in endothelial cells by thromboxane A_2. *Circ Res* 2000; 87: 739–745.

24 Hirata T, Ushikubi F, Kakizuka A, Okuma M, Narumiya S. Two thromboxane receptor isoforms in human platelets. Opposite coupling to adenylyl cyclase with different sensitivity to Arg^{60} to Leu mutation. *J Clin Invest* 1996; 97: 949–956.

25 Habib A, FitzGerald GA, Maclouf J. Phosphorylation of the thromboxane receptor α, the predominant isoform expressed in human platelets. *J Biol Chem* 1999; 274: 2645–2651.

26 Ushikubi F, Hirata M, Narumiya S. Platelet prostaglandin receptors. In: von Bruchhausen F, Walter U eds. *Platelets and their factors.* Berlin, Germany: Springer 1997; 135–154.

27 D'Angelo DD, Eubank JJ, Davis MG, Dorn GW II. Mutagenic analysis of the platelet thromboxane receptor cysteines. Roles in ligand binding and receptor-effector coupling. *J Biol Chem* 1996; 271: 6233–6240.

28 Dorn GW II, Davis MG, D'Angelo DD. Structural determinants for agonist binding affinity to thromboxane/prostaglandin endoperoxide (TP) receptors. Analysis of chimeric rat/human TP receptors. *J Biol Chem* 1997; 272: 12399–12405.

29 Funk CD, Furci L, Moran N, FitzGerald GA. Point mutation in the seventh hydrophobic domain of the human thromboxane A_2 receptor allows discrimination between agonist and antagonist binding sites. *Mol Pharmacol* 1993; 44: 934–939.

30 Zhou H, Yan F, Yamamoto S, Tai HH. Phenylalanine 138 in the second intracellular loop of human thromboxane receptor is critical for receptor-G-protein coupling. *Biochem Biophys Res Commun* 1999; 264: 171–175.

31 Spurney RF, Coffman TM. The C-terminus of the thromboxane receptor contributes to coupling and desensitization in a mouse mesangial cell line. *J Pharmacol Exp Ther* 1997; 283: 207–215.

32 Nusing RM, Hirata M, Kakizuka A, Eki T, Ozawa K, Narumiya S. Characterization and chromosomal mapping of the human thromboxane A_2 receptor gene. *J Biol Chem* 1993; 268: 25253–25259.

33 D'Angelo DD, Davis MG, Houser WA, Eubank JJ, Ritchie ME, Dorn GW II. Characterization of the 5′ end of human thromboxane receptor gene. Organizational analysis and mapping of protein kinase C-responsive elements regulating expression in platelets. *Circ Res* 1995; 77: 466–474.

34 Le TH, Thomas DW, Coffman TM, Pfister SL. Vascular unresponsiveness to thromboxane A_2 (TxA_2) is associated with a polymorphism at the thromboxane (TP) receptor locus. *Hypertension* 1999; 34: 349.

35 Hirata T, Kakizuka A, Ushikubi F, Fuse I, Okuma M, Narumiya S. Arg^{60} to Leu mutation in the human thromboxane A_2 receptor in a dominantly inherited bleeding disorder. *J Clin Invest* 1994; 94: 1662–1667.

36 Unoki M, Furuta S, Onouchi Y et al. Association studies of 33 single nucleotide polymorphisms (SNPs) in 29 candidate genes for bronchial asthma: positive association a T924C polymorphism in the thromboxane A_2 receptor gene. *Hum Genet* 2000; 106: 440–6.

37 Namba T, Sugimoto Y, Hirata M et al. Mouse thromboxane A_2 receptor cDNA cloning, expression and northern blot analysis. *Biochem Biophys Res Commun* 1992; 184: 1197–1203.

38 Thomas DW, Mannon RB, Latour A et al. Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A_2. *J Clin Invest* 1998; 102: 1994–2001.

39 Rocca B, Loeb AL, Strauss JF III et al. Directed vascular expression of the thromboxane A_2 receptor results in intrauterine growth retardation. *Nat Med* 2000; 6: 219–221.

40 Nowak J, Murray JJ, Oates JA, FitzGerald GA. Biochemical evidence of a chronic abnormality in platelet and vascular function in healthy individuals who smoke cigarettes. *Circulation* 1987; 76: 6–14.

41 Alessandrini P, McRae J, Feman S, FitzGerald GA. Thromboxane biosynthesis and platelet function in type-I diabetes mellitus. *N Engl J Med* 1988; 319: 208–212.

42 Paul BZS, Jin J, Kunapuli SP. Molecular mechanism of thromboxane A_2-induced platelet aggregation. Essential role for $P2T_{AC}$ and α_{2A} receptors. *J Biol Chem* 1999; 274: 29108–29114.

43 Shenker A, Goldsmith P, Unson CG, Spiegel AM. The G protein coupled to the thromboxane A_2 receptor in human platelets is a member of the novel Gq family. *J Biol Chem* 1991; 266: 9309–9313.

44 Knezevic I, Borg C, Le Breton GC. Identification of Gq as one of the G proteins which copurify with human platelet thromboxane A_2/prostaglandin H_2 receptors. *J Biol Chem* 1993; 268: 26011–26017.

45 Offermanns S, Laugwitz KL, Spicher K, Schultz G. G proteins of the G_{12} family are activated via thromboxane A_2 and thrombin receptors in human platelets. *Proc Natl Acad Sci USA* 1994; 91: 504–508.

46 Offermanns S, Simon MI. $G\alpha_{15}$ and $G\alpha_{16}$ couple a wide variety of receptors to phospholipase C. *J Biol Chem* 1995; 270: 15175–15180.

47 Moran N, FitzGerald GA. Purification of an abundant guanine-nucleotide binding (G) protein linked to human platelet (HP) prostaglandin (PGH_2)/thromboxane (Tx) receptor. *Circulation* 1990; 82: 461.

48 Vezza R, Habib A, FitzGerald GA. Differential signalling by the thromboxane receptor isoforms *via* the novel GTP-binding protein, Gh. *J Biol Chem* 1999; 274: 12774–12779.

49 Fuse I, Mito M, Hattori A et al. Defective signal transduction induced by thromboxane A_2 in a patient with a mild bleeding disorder: impaired phospholipase C activation despite normal phospholipase A_2 activation. *Blood*, 1993; 81: 994–1000.

Cambridge Books Online © Cambridge University Press, 2009

50 Dhanasekaran N, Dermott JM. Signalling by the G_{12} class of G proteins. *Cell Signal* 1996; 8: 235–245.
51 Arita H, Nakano T, Hanasaki K. Thromboxane A_2: its generation, and role in platelet activation. *Prog Lipid Res* 1989; 28: 273–301.
52 Brass LF, Shaller CC, Belmonte EJ. Inositol 1,4,5-triphosphate induced granule secretion in platelets. Evidence that the activation of phospholipase C mediated by platelet thromboxane receptors involves a guanine nucleotide binding protein-dependent mechanism distinct from that of thrombin. *J Clin Invest* 1987; 79: 1269–1275.
53 Ushikubi F, Nakamura K, Narumiya S. Functional reconstitution of platelet thromboxane A_2 receptors with G_q and G_{i2} in phospholipid vesicles. *Mol Pharmacol* 1994; 46: 808–816.
54 Murray R, Shipp E, FitzGerald GA. Prostaglandin endoperoxide/thromboxane A_2 receptor desensitization. Cross-talk with adenylate cyclase in human platelets. *J Biol Chem* 1990; 265: 21670–21675.
55 FitzGerald GA. Mechanisms of platelet activation: thromboxane A_2 as an amplifying signal for other agonists. *Am J Cardiol* 1991; 68: 11B-15B.
56 Elalamy I, Emadi S, Vargaftig B, Hatmi M. Signal transduction involved in the platelet adenylate cyclase sensitization associated with PGH_2/TxA_2 receptor desensitization. *Br J Haematol* 1997; 99: 190–196.
57 Fulon I, Bachir D, Galacteros F, Maclouf J. Increased in vivo production of thromboxane in patients with sickle cell disease is accompanied by an impairment of platelet functions to the thromboxane agonist U46619. *Arterioscler Thromb* 1993; 13: 421–426.
58 Vezza R, Nenci GG, Gresele P. Thromboxane synthase inhibitors suppress more efficiently the aggregation of thromboxane receptor-desensitized platelets than that of normal platelets: role of adenylate cyclase up-regulation. *J Pharmacol Exp Ther* 1995; 275: 1497–1505.
59 Habib A, Vezza R, Creminon C, Maclouf J, FitzGerald GA. Rapid, agonist-dependent phosphorylation in vivo of human thromboxane receptor isoforms. Minimal involvement of protein kinase C. *J Biol Chem* 1997; 272: 7191–7200.
60 Kinsella BT, O'Mahony DJ, FitzGerald GA. Phosphorylation and regulated expression of the human thromboxane A_2 receptor. *J Biol Chem* 1994; 269: 29914–29919.
61 Yukawa M, Yokota R, Eberhardt RT, von Andrian L, Ware JA. Differential desensitization of thromboxane A_2 receptor subtypes. *Circ Res* 1997; 80: 551–556.
62 Walsh MT, Foley JF, Kinsella BT. The α, but not β, isoform of the human thromboxane A_2 receptor is a target for prostacyclin-mediated desensitization. *J Biol Chem* 2000; 275: 20412–20423.
63 Spurney RF. Role of C-terminal serines in desensitization and phosphorylation of the mouse thromboxane receptor. *J Biol Chem* 1998; 273: 28496–28503.
64 Wang GR, Zhu Y, Halushka PV, Lincoln TM, Mendelsohn ME. Mechanism of platelet inhibition by nitric oxide: in vivo phosphorylation of thromboxane receptor by cyclic GMP-dependent protein kinase. *Proc Natl Acad Sci USA* 1998; 95: 4888–4893.
65 Parent JL, Labrecque P, Orsini MJ, Benovic JL. Internalization of the TxA_2 receptor α and β isoforms. Role of the differentially spliced COOH terminus in agonist-promoted receptor internalization. *J Biol Chem* 1999; 274: 8941–8948.
66 Weyrich AS, Dixon DA, Pabla R et al. Signal-dependent translation of a regulatory protein, Bcl-3, in activated human platelets. *Proc Natl Acad Sci USA* 1998; 95: 5556–5561.
67 Nakajima M, Yamamoto M, Ushikubi F, Okuma M, Fujiwara M, Narumiya S. Expression of thromboxane A_2 receptor in cultured human erythroleukemia cells and its induction by 12–O-tetradecanoyl phorbol 13-acetate. *Biochem Biophys Res Commun* 1989; 158: 958–965.
68 Matsuda K, Mathur R, Duzic E, Halushka PV. Androgen regulation of thromboxane A_2/prostaglandin H_2 receptor expression in human erythroleukemia cells. *Am J Physiol* 1993; 265: E9228–E9234.
69 Ajayi AA, Mathur R, Halushka PV. Testosterone increases human platelet thromboxane A_2 receptor density and aggregation responses. *Circulation* 1995; 91: 2742–2747.
70 Pratico D, FitzGerald GA. Testosterone and thromboxane: of muscles, mice, and men. *Circulation* 1995; 91: 2694–2698.
71 Braden G, Knapp HR, FitzGerald GA. Suppression of eicosanoid formation during coronary angioplasty by fish oil or aspirin. *Circulation* 1991; 84: 679–685.
72 Cheng Y, Sagel S, Li W, FitzGerald GA. Thromboxane receptor (TP) activation and the proliferative response to catheter-induced vascular injury in the mouse. Winter Eicosanoid Conference 2000, Baltimore, MD (Abstract).
73 Pratico D, Cheng Y, FitzGerald GA. TP or not TP: primary mediators in a close runoff? *Arterioscler Thromb Vasc Biol* 2000; 20: 1695–1698.
74 Cayatte AJ, Du Y, Oliver-Krasinski J, Lavielle G, Verbeuren TJ, Cohen RA. The thromboxane receptor antagonist S18886 but not aspirin inhibits atherogenesis in apo-E-deficient mice: evidence that eicosanoids other than thromboxane contribute to atherosclerosis. *Arterioscler Thromb Vasc Biol* 2000; 20: 1724–1728.
75 Nakagawa O, Tanaka I, Usui T et al. Molecular cloning of human prostacyclin receptor cDNA and its gene expression in the cardiovascular system. *Circulation* 1994; 90: 1643–1647.
76 Boie Y, Rushmore TH, Darmon-Goodwin A et al. Cloning and expression of a cDNA for the human prostanoid IP receptor. *J Biol Chem* 1994; 269: 12173–12178.
77 Sasaki Y, Takahashi T, Tanaka I et al. Expression of prostacyclin receptor in human megakaryocytes. *Blood* 1997; 90: 1039–1046.
78 Neri Serneri GG, Modesti PA, Fortini A, Abbate R, Lombardi A, Gensini GF. Reduction in prostacyclin platelet receptors in active spontaneous angina. *Lancet* 1984; 2: 838–841.
79 Katsuyama M, Sugimoto Y, Namba T et al. Cloning and expression of a cDNA for the human prostacyclin receptor. *FEBS Lett* 1994; 344: 74–78.

Cambridge Books Online © Cambridge University Press, 2009

80 Smyth EM, Nestor PV, FitzGerald GA. Agonist-dependent phosphorylation of an epitope-tagged human prostacyclin receptor. *J Biol Chem* 1996; 271: 33698–33704.
81 Hayes JS, Lawler OA, Walsh MT, Kinsella BT. The prostacyclin receptor is isoprenylated. Isoprenylation is required for efficient receptor-effector coupling. *J Biol Chem* 1999; 274: 23707–23718.
82 Zhang Z, Austin SC, Smith, EM. Glycosylation of the human prostacyclin receptor: role in ligand binding and signal transduction. *Mol Pharm* 2001; 60: 480–487.
83 Kahn NN, Bauman WA, Sinha AK. Loss of high affinity prostacyclin receptors in platelets and the lack of prostaglandin-induced inhibition of platelet-stimulated thrombin generation in subjects with spinal cord injury. *Proc Natl Acad Sci USA* 1996; 93: 245–249.
84 Ogawa Y, Tanaka I, Inoue M et al. Structural organization and chromosomal assignment of the human prostacyclin receptor gene. *Genomics* 1995; 27: 142–148.
85 Murata T, Ushikubi F, Matsuoka T et al. Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature* 1997; 388: 678–682.
86 Haslam R, Davidson MML, Davies T, Lynham JA, McCleghan MD. Regulation of blood platelet function by cyclic nucleotides. *Adv Cyclic Nucleotide Res* 1978; 9: 533–552.
87 Manganello JM, Djellas Y, Borg C, Antonakis K, Le Breton GC. Cyclic AMP-dependent phosphorylation of thromboxane A_2 receptor-associated $G\alpha_{13}$. *J Biol Chem* 1999; 274: 28003–28010.
88 Oka M, Negishi M, Nishigaki N, Ichikawa A. Two types of prostacyclin receptor coupling to stimulation of adenylate cyclase and phosphotidylinositol hydrolysis in a cultured mast-cell line, BNU-2C13 cells. *Cell Signalling* 1993; 5: 643–650.
89 Vassaux G, Gaillard D, Ailhaud G, Nigrel R. Prostacyclin is a specific effector of adipocite cell differentiation. Its dual role as a cAMP-elevating and a Ca^{2+}-elevating agent. *J Biol Chem* 1992; 267: 11902–11907.
90 Giovannazzi S, Accomazzo MR, Letari O, Oliva D, Nicosia S. Internalization and down-regulation of the prostacyclin receptor in human platelets. *Biochem J* 1997; 325: 71–77.
91 Fisch A, Tobusch K, Veit K, Darius H. Prostacyclin receptor desensitization is a reversible phenomenon in human platelets. *Circulation* 1997; 96: 756–760.
92 Smyth EM, Li WH, FitzGerald GA. Phosphorylation of the prostacyclin receptor during homologous desensitization. A critical role for protein kinase C. *J Biol Chem* 1998; 273: 23258–23266.
93 Nilius SM, Hasse A, Kuger P, Schror K, Meyer-Kirchrath J. Agonist-induced long-term desensitization of the human prostacyclin receptor. *FEBS Lett* 2000; 484: 211–216.
94 Smyth EM, Austin SC, Reilly MP, FitzGerald GA. Internalization and sequestration of the human prostacyclin receptor. *J Biol Chem* 2000; 275: 32037–32045.
95 Kunapuli SP, Mao FG, Bastepe M et al. Cloning and expression of a prostaglandin E receptor EP3 subtype from human erythroleukemia cells. *Biochem J* 1994; 298: 263–267.
96 Matthews JS, Jones RL. Potentiation of aggregation and inhibition of adenylate cyclase in human platelets by prostaglandin E analogues. *Br J Pharmacol* 1993; 108: 363–869.
97 Kotani M, Tanaka I, Ogawa T et al. Molecular cloning and expression of multiple isoforms of human prostaglandin E receptor EP3 subtype generated by alternative messenger RNA splicing: multiple second messenger systems and tissue specific distributions. *J Pharmacol Exp Ther* 1995; 48: 869–879.
98 Regan JW, Bailey TJ, Donello JE et al. Molecular cloning and expression of human EP3 receptors: evidence of three variants with differing carboxyl termini. *Br J Pharmacol* 1994; 112: 377–385.
99 Schmid A, Thierauch KH, Schileuning WD, Dinter H. Splice variants of the human EP3 receptor for prostaglandin E_2. *Eur J Biochem* 1995; 228: 23–30.
100 Paul BZS, Ashby B, Sheth SB. Distribution of prostaglandin IP and EP receptor subtypes and isoforms in platelets and human umbilical artery smooth muscle cells. *Br J Haematol* 1998; 102: 1204–1211.
101 Ma H, Hara A, Xiao CY et al. Increased bleeding tendency and decreased susceptibility to thromboembolism in mice lacking the prostaglandin E receptor subtype EP3. *Circulation* 2001; 104: 1176–1180.
102 Kotani M, Tanaka I, Ogawa T et al. Structural organization of the human prostaglandin EP3 receptor subtype gene (PTGER3). *Genomics* 1997; 40: 425–434.
103 Audoly L, Breyer RM. The second extracellular loop of the prostaglandin EP3 receptor is an essential determinant of ligand selectivity. *J Biol Chem* 1997; 272: 13475–13478.
104 Ushikubi F, Segi E, Sugimoto Y et al. Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP3. *Nature* 1998; 395: 281–284.
105 Gray S, Heptinstall S. The effects of PGE_2 and CL115,347, an antihypertensive PGE_2 analogue, on human blood platelets behavior and vascular contractility. *Eur J Pharmacol* 1985; 114: 129–137.
106 McIntyre DE, Gordon JL. Calcium-dependent stimulation of platelet aggregation by PGE_2. *Nature* 1975; 258: 337–339.
107 Gresele P, Blockmans D, Deckmyn H, Vermylen J. Adenylate cyclase activation determines the effect of thromboxane synthase inhibitors on platelet aggregation in vitro. Comparison of platelets from responders and non-responders. *J Pharmacol Exp Ther* 1988; 246: 301–307.
108 Mao GF, Jin JG, Bastepe M, Ortiz-Vega S, Ashby B. Prostaglandin E_2 both stimulates and inhibits adenyl cyclase on platelets: comparison of effects on cloned EP4 and EP3 prostaglandin receptor subtypes. *Prostaglandins* 1996; 52: 175–185.
109 Wu H, James-Kracke MR, Halenda SP. Direct relationship between intracellular calcium mobilization and phospholipase D activation in prostaglandin E-stimulated human erythroleukemia cells. *Biochemistry* 1992; 31: 3370–3377.
110 Hasegawa H, Negishi M, Ichikawa A. Two isoforms of the prostaglandin E receptor EP3 subtype different in agonist-

Cambridge Books Online © Cambridge University Press, 2009

independent constitutive activity. *J Biol Chem* 1996; 271: 1857–1860.

111 Namba T, Sugimoto Y, Negishi M et al. Alternative splicing of C-terminal tail of prostaglandin E receptor subtype EP3 determines G-protein specificity. *Nature* 1993; 365: 166–170.

112 An S, Yang J, So SW, Zeng L, Goetzl EJ. Isoforms of the EP3 subtype of human prostaglandin E_2 receptor transduce both intracellular calcium and cAMP signals. *Biochemistry* 1994; 33: 14496–14502.

113 Jin J, Mao GF, Ashby B. Constitutive activity of human prostaglandin E receptor EP3 isoform. *Br J Pharmacol* 1997; 121: 317–323.

114 Negishi M, Sugimoto Y, Irie A, Narumiya S, Ichikawa A. Two isoforms of prostaglandin E receptor EP3 subtype. Different COOH-terminal domains determine sensitivity to agonist-induced desensitization. *J Biol Chem* 1993; 268: 9517–9521.

115 Hirata M, Kakizuka A, Aizawa M, Ushikubi F, Narumiya S. Molecular characterization of a mouse prostaglandin D receptor and functional expression of the cloned gene. *Proc Natl Acad Sci USA* 1994; 91: 11192–11196.

116 Boie Y, Sawyer N, Slipetz DM, Metters KM, Abramovitz M. Molecular cloning and characterization of the human prostanoid DP receptor. *J Biol Chem* 1995; 270: 18910–18916.

117 Matsuoka T, Hirata M, Tanaka H et al. Prostaglandin D_2 as a mediator of allergic asthma. *Science* 2000; 287: 2013–2017.

118 Austin SC, Funk CD. Insight into prostaglandin, leukotriene, and other eicosanoid functions using mice with targeted gene disruptions. *Prostaglandins Other Lipid Mediators* 1999; 58: 231–252.

119 Yoshimura S, Mizuno Y, Kimura K et al. Prostaglandin D_2 receptor of mastocytoma P-815 cells – possible regulation by phosphorylation and dephosphorylation. *Biochim Biophys Acta* 1989; 981: 69–76.

Cambridge Books Online © Cambridge University Press, 2009

11

Platelet receptors: collagen

Pia R-M. Siljander and Richard W. Farndale

University of Cambridge, Department of Biochemistry, Cardiovascular Biology Section, Cambridge, UK

Introduction

Platelet–collagen interaction is fundamental to both normal haemostasis and pathological thrombotic incidents. In recent years the complexity of platelet collagen receptors and their interplay has become better understood. However, further work is needed before the specific recognition sequences in collagens for each receptor are identified and the events leading from initial contact to primary adhesion and full-blown platelet activation become mapped. To achieve this will require knowledge of the relevant collagen receptors themselves, the signalling pathways they activate and the vascular collagens with which they interact. It is also becoming clear that accessory molecules on the platelet surface, together with adhesive proteins that bind both collagen and the platelet, can each modulate the interaction without being direct collagen receptors themselves. Finally, we envisage that the importance of employing experimental conditions which more accurately reflect the platelet–collagen interaction in vivo will become more widely recognised. Thus, the presentation of collagen in triple-helical conformation, as polymeric fibres rather than monomers, immobilized on a surface rather than in suspension, and finally under shear conditions which represent blood flow, will have a strong impact on the final understanding of collagen as an adhesive substrate and a platelet agonist. We aim here to pool current awareness of the various aspects of the platelet-collagen interaction introduced above. For recent reviews, see[1–8,*1,*2].

Early work, using tissue extracts in turbidimetric aggregometry, established the importance of collagen as a platelet agonist[9–11]. This method provides limited insight into the different adhesive and activatory properties of collagen, as it measures solely the upregulation of the fibrinogen receptor, αIIbβ3, to which secondary stimuli contribute together with primary signals from collagen. However, such studies demonstrated that both triple-helical conformation and higher-order structure in collagens are necessary to support platelet aggregation[12–15]. Subsequent work, using platelet adhesion to immobilized cyanogen bromide fragments of collagens I and III, located adhesive and activatory sites at several different positions within the tropocollagen molecule, and established a set of recognition sites essential for primary platelet adhesion[16–19]. From this basis, candidate sequences were identified and triple-helical peptides synthesized[20–22] and used to locate the site of interaction in collagen with the first collagen receptors identified, such as $\alpha2\beta1$[23–28]. To date, little equivalent information is available for the other well-characterized platelet collagen receptors, CD36 and Glycoprotein (Gp) VI (see pp. 162–163).

The future elucidation of the detail of the platelet–collagen interaction will involve peptide-binding studies, purification and cloning of receptors, knockout technology and understanding how the several receptors coordinate platelet adhesion and signalling.

Collagens of the vascular system

Nine of the different human collagens identified so far[21–*3], types I, III–VI, VIII and XII–XIV, have been located in the vasculature, where the fibrillar collagens I, III and V are the major species, and the network-forming collagen type IV occurs in the subendothelial basement membrane[29,30]. Collagens form 20–40% of total protein in aorta and other arteries[31]. Furthermore, a developing atherosclerotic plaque becomes enriched in both type I and type III collagens[32]. Vessel wall collagens provide an important primary stimulus for platelet adhesion and activation upon exposure to blood flow by injury or disease. Although collagens I and III are revealed mainly when injury reaches the media

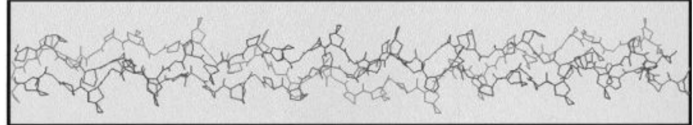
or adventitia, in some vessels they also occur in the subendothelium[33,34]. Not surprisingly, these collagens, including type IV, are reported to have the highest platelet reactivity (although XIII and XIV have yet to be evaluated), each able to induce adhesion and aggregation under both static and flow conditions[1,35,36]. In contrast, collagen V, which is important in the organisation of composite fibres with other collagens, has been reported to support only static platelet adhesion[35].

Collagens are defined by their common primary structure, a sequence of repeating G–X–X′ triplets, where G is glycine, X is often proline (P) and X′, hydroxyproline (O). The sequence GPO represents about 10% of the fibrous collagens I and III. Collagen I consists of two α_1(I)-chains and one α_2(I)-chain, whereas collagen III is a disulfide-bonded homotrimer of α_1(III)-chains. Each of the α-chains forms a left-handed helix, of which a G–X–X′ triplet represents one turn, and together they comprise a right-handed superhelix (polyproline helix II), forming the tropocollagen molecule. The small amino acid G occupies the axial position of the triple-helix, while X and X′ face the external milieu[37–40]. Each chain in collagens I and III possesses a triple-helical domain of ~1000 amino acids (length 300 nm, diameter 1.5 nm) with short non-helical N- and C-terminal telopeptide extensions. In non-fibrillar collagens, the triple-helical parts may be flanked by other domains, such as an A-domain fold like that of von Willebrand factor (vWf)[41].

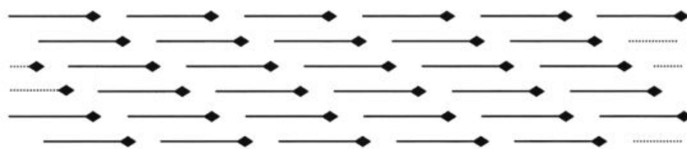
The preservation of collagen's native conformation depends strongly on its source and method of isolation[42]. As a structural component of tissue, existing generally in complex with other poorly soluble molecules, collagen has often been isolated using proteases. While the triple-helical domains resist digestion except by specific collagenases[43,44], the telopeptides are susceptible to proteolysis, e.g. by pepsin. Thus, 'native' fibres have often been prepared using proteolytically modified collagen monomers. However, the initiation of collagen fibril formation is seriously impaired by the removal of telopeptides[45–49], as they contain those lysine and hydroxylysine residues which become covalently cross-linked to stabilise the fibril[48].

Native collagen fibres are formed by arrangement of tropocollagen molecules side-by-side and end-to-end in a quarter-staggered array[50] (Fig. 11.1, see colour plate). The polymeric organization of collagen fibrils can be seen as a typical striated (670 ± 30 Å) structure in transmission electron microscopy. In vivo collagen fibres can be hybrids of different collagen types, e.g. I with V or III, and connective tissue components such as proteoglycans associate with the fibres, affecting their fibril formation and binding properties[51]. It should be noted that many matrix components, e.g. laminin and plasma proteins (vWf, fibronectin, thrombospondin) can also bind both collagen and platelets. This complexity obscures the understanding of the primary platelet–collagen interaction.

Triple-helical collagen monomer



Monomers assemble to form fibril



Native collagen fibre

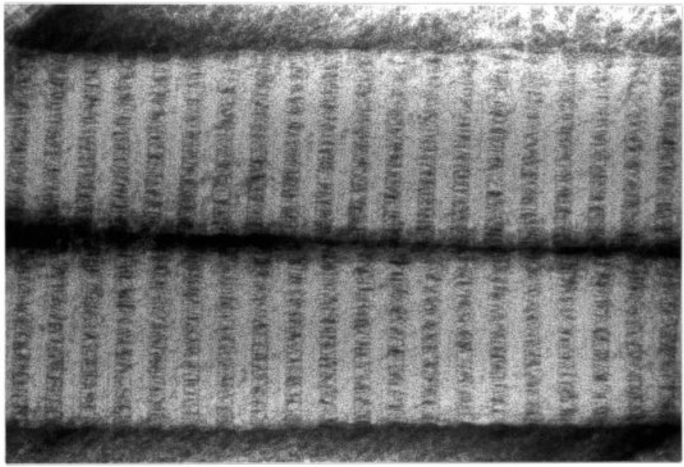


Fig. 11.1 (see also colour plate). Illustrates the hierarchical structure of collagen: the monomer is represented using the crystal coordinates of $[GPO]_{12}$, obtained from 2clg.pdb with its individual alpha chains shown in different colours, and the proline and hydroxyproline residues protruding into the external medium. The tropocollagen monomers assemble head-to-tail in a quarter-staggered array into fibrils, and their organization into fibres is clearly visualized by transmission electron microscopy. ◆ indicates the N-terminus of the tropocollagen molecule. (Transmission electron microscopy, courtesy of M. Hess, University of Helsinki.)

Understanding of the molecular structure of collagen, from simple linear sequences to the correct spatial presentation of an array of receptor-specific ligands, of different collagen species and their capacity to associate with other

Cambridge Books Online © Cambridge University Press, 2009

molecules, are each essential for the resolution of the platelet–collagen interaction. New data in this rapidly developing area drives the evolution of the simple two-site, two-step concept[3,18,52,53] towards a multisite, co-operative adhesive and signalling model (see pp. 165–168).

Importance of platelet–collagen interaction in haemostasis and thrombosis

Evidence for a role for collagen in the haemostatic response has hitherto been indirect. A very few patients affected by bleeding disorders were found to be defective in the expression of the platelet collagen receptors, or to express autoantibodies against them[54–62]. Some of these anomalies have been transient[63]. In each case, however, platelets were either refractory to stimulation by collagen, or lacked the capacity to adhere to collagen. The absence of specific tools to antagonize collagen receptors in vivo has hindered direct proof of their importance. Defective collagen synthesis is also known to impair haemostasis[64–66].

The platelet–collagen interaction has been shown to be a cornerstone for deposition of platelets in human atheromatous coronary artery explants[32]. It is increasingly accepted that collagen exposed during rupture or fissure of atherosclerotic plaque leads to platelet adhesion and activation. Exposure of subendothelial collagens in the vessel wall cannot be avoided during bypass grafting or angioplasty, and is considered to precipitate acute thrombotic episodes that accompany both procedures[67]. As antiplatelet therapy at the time of angioplasty prolongs vessel patency, it seems plausible that activation of platelets by agonists including collagen may stimulate the release or generation of mitogens and chemotactic substances, such as PDGF or thrombin, that may provoke restenosis[68].

Activation of platelets contributes to thrombotic events either through direct thrombus formation or through the procoagulant response. Two linked, silent polymorphisms in the $\alpha2$ gene (C807T and G873A) correlate with the expression of the collagen receptor $\alpha2\beta1$ on the platelet surface[69], which varies by a factor of about 4[70,71]. The 807T/873A (high receptor expression) polymorphism has been associated with increased risk of stroke and myocardial infarction[72–76], strongly supporting the proposed importance of collagen receptors in the sequelae of atherosclerosis[77]. Conversely, low $\alpha2\beta1$ density, correlating with 807C, has been linked to bleeding tendency[78]. Other reports show no such correlation, which may depend on the exact study population and the clinical endpoint being investigated[79–81,*4–*7]. The A1648G (HPA-5) polymorphism correlated with increased risk of cardiovascular events in otherwise low-risk groups[82], whereas the SIT(a) C2531T dimorphism of the $\alpha2$ gene leads to impaired response to collagen[83]. A recent study suggested that GpVI-depletion in mice provided protection against lethal thromboembolism[84]. Opinion is moving in favour of platelet collagen receptors as important mediators of thrombosis, and therefore as targets for antithrombotic therapy[4].

Platelet receptors for collagen

At present, only two receptors are both sequenced and understood in some functional detail, namely integrin α2b1 and GpVI. In addition, the sequence of two other receptors, perhaps of lesser significance, is known: CD36, and the p65 collagen I receptor. Binding motifs within collagen are being elucidated at present for $\alpha2\beta1$, for GpVI and for a less well-characterized receptor, specific for collagen III (Table 11.1).

Several other platelet proteins, such as cC1qR, have been proposed as collagen receptors[5,6,*8]. Many of these, including type III collagen-binding protein (TIIICBP) and p65, await independent verification. Collagen receptor function may involve regulatory membrane receptors or other accessory molecules, and examples of these are described below. It should be noted that the collagen-binding tyrosine kinase receptors, the discoidin domain receptors, DDR1 and DDR2[85], have not been located on platelets.

The integrin $\alpha2\beta1$ (Gp Ia/IIa, CD49b/CD29, VLA_2)

In addition to other $\beta1$ integrins ($\alpha5\beta1$ and $\alpha6\beta1$, the receptors for fibronectin and laminin, respectively) platelets express $\alpha2\beta1$[86], which is also the major collagen receptor in many other cell types[87]. In contrast to the single $\alpha2$ isoform, several different $\beta1$ gene products exist, and the A isoform predominates in platelets, although the C-isoform has been located by RT-PCR[88]. The reported receptor densities for $\alpha2\beta1$ vary between individuals from about 800 to 3500 copies per platelet[70,71,89]. $\alpha2\beta1$-deficient patients[54,63] have significant bleeding problems, and their platelets display impaired spreading upon adhesion to collagen[55]. So far, no $\alpha2$ knockout mice have been reported, but $\beta1$ knockouts exhibit early embryonic lethality[*9]. As yet, other collagen-binding integrins, $\alpha1\beta1$, $\alpha10\beta1$ and $\alpha11\beta1$, have not been located on platelets. In other cells, $\alpha2\beta1$ can also bind the physiological ligands, laminin, E-cadherin and echovirus-1[90–93], but the selectivity of $\alpha2\beta1$ for particular ligands is poorly understood. Integrins are α/β heterodimers, and subunit association, through electrostatic interac-

Cambridge Books Online © Cambridge University Press, 2009

Table 11.1. Properties of established collagen receptors

Receptor	kDa in gel (reduced)	kDa (polypeptide)	Glycosylation	Copy number	Accession number	Recognition sequence
$\alpha2/\beta1$	165/130	$\alpha2$ 126 $\beta1$A 86 $\beta1$C 89	10 N, O? 12 N, O? 12 N, O?	800–3500	P17301 P05556 P05556	GFOGER and homologues $\alpha1$(I) chain
GpVI	62	35	1 N, O^{++}	~1000	Q9UIF2	$[GPO]_n$
CD36	88	53	10 N, O^{+}	20 000	P16671	?
TIIICBP	68/72	?	no	?	–	KOGEOGPK $\alpha1$(III) chain
p65	65	54	2 N?, O?	?	None assigned	?

Notes:
Accession numbers are for Swiss-Prot, except for GpVI which is from TSEIIBL.
See text for references.

tion between their transmembrane regions, is necessary for both cell-surface expression and for competence to bind ligands. The general structure of integrins has recently been reviewed[87].

The $\alpha2$ subunit inserted (I-) domain, which is homologous with the A-domains of vWf, is the site of interaction of $\alpha2\beta1$ with collagen. The integrin α subunit has been modelled as a seven bladed β-propeller[94,*10], and the I-domain is located above blades 2 and 3. Collagen binding centres upon a divalent cation co-ordinated within the metal ion-dependent adhesion site (MIDAS) in the I-domain. Mg^{2+} is the physiological ion, though in vitro, several others, notably Mn^{2+}, may augment the adhesive process[87]. Lately, Ni^{2+} has also been reported to enhance collagen-induced platelet activation[95]. However, the role of divalent cations is not restricted to the MIDAS. $\alpha2\beta1$ requires micromolar Ca^{2+} to bind collagen[96], possibly, like αIIb$\beta3$[97,98], to stabilize the association between subunits. The $\alpha2$-subunit contains three Ca^{2+}-binding sites, each with characteristic DXXXDXXXD motif. Recently, these structures were suggested to resemble β-hairpin loops rather than EF-hands[99]. Expression of partial $\alpha2$ constructs suggests that the first Ca^{2+}-binding loop (in blade 4 of the putative β-propeller) may enhance the binding competence of the integrin[100]. In contrast, millimolar Ca^{2+} levels inhibit $\alpha2\beta1$ function[101]. As the large Ca^{2+} ion will not fit into the $\alpha2$ I-domain MIDAS[102], it is plausible that an inhibitory Ca^{2+}-binding site resides in the β-subunit of the integrin. Other $\alpha2\beta1$-associated Ca^{2+}-binding molecules may also contribute to Ca^{2+}-dependent effects. In summary, the divalent cation-dependence is derived from three different sites within the integrin. Finally, it is of interest that $\alpha2\beta1$ has been reported to exhibit cation-independent binding of echovirus-1 and snake venom rhodocytin[103,104].

The major collagen motif binding to the $\alpha2$ I-domain MIDAS is the sequence GFOGER[27], residues 502 to 507 of the $\alpha1$(I) chain. This sequence, as a triple-helical synthetic peptide, has been co-crystallized with recombinant I-domain, allowing the first structural analysis of an integrin–ligand complex[102]. Site-directed mutagenesis[91,105,106] and the use of inhibitory monoclonal antibodies[93,107] previously identified sequences close to the MIDAS crucial for collagen binding. The co-crystal structure confirms direct co-ordination of the MIDAS cation by the GFOGER glutamate residue (recently reviewed[108]), and also provides evidence for two conformations of the I-domain, possibly representing either inside-out (affinity regulation) or outside-in signalling (see Fig. 11.2, see colour plate). Only two strands of the triple-helix interact with the I-domain in the crystal structure[102] and thus, homotrimeric peptides containing the equivalent sequence from $\alpha2$(I), GPOGES, are incapable of binding $\alpha2\beta1$ (authors unpublished data). A similar sequence, GLOGER, has also been located within collagen I as an $\alpha2\beta1$-recognition motif, along with a lower-affinity motif, GASGER[28]. However, GFOGER-containing peptides can inhibit platelet adhesion to immobilized collagen I, suggesting that other sequences are of lower affinity[27]. In collagen IV, GFOGER is the major site of interaction with $\alpha2\beta1$, although other higher affinity sequences are present for $\alpha1\beta1$[27,109], another major collagen-binding integrin not found in platelets. Previous reports identified the sequence DGEA, close to GFOGER in the collagen I $\alpha1$ chain, as an $\alpha2\beta1$-recognition motif for platelets[110]. These data have not been confirmed, and triple-helical peptides containing DGEA do not support $\alpha2\beta1$ or platelet binding[26]. Despite this, DGEA is reported to inhibit adhesion of other cell types[111–113].

Cambridge Books Online © Cambridge University Press, 2009

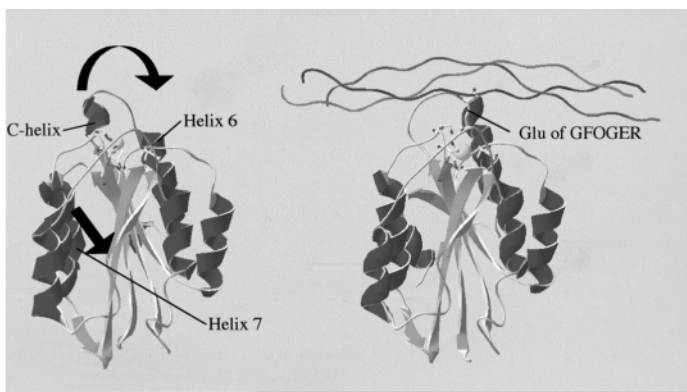


Fig. 11.2 (see also colour plate). Shows the differing conformation of the free integrin $\alpha 2$ I-domain compared with that bound by collagen. As indicated by the black arrows, the resting I-domain moves upon ligation by the collagen- like peptide. The C-helix unwinds and is added to the top of helix 6. Helix 7 moves axially towards the base of the I-domain. The I-domain construct contains a truncated helix 7, the native form of which, in the active conformation, would protrude from the I-domain and alter its relationship with the rest of the $\alpha 2$ subunit. The metal ion in the MIDAS is shown as a yellow sphere. Crystal coordinates, free I domain, 1aox,.pdb; peptide-I-domain complex, 1dzi.pdb. (Molecular graphics courtesy of Dr D. Tuckwell, University of Manchester.)

Glycoprotein VI

GpVI (p62) has recently been cloned and expressed by several groups[114–119]. It is most closely related to the immunoglobulin superfamily natural killer receptor, 1NKR, and to Fcα-R. Thus far, GpVI has been located only on platelets and late megakaryocytes[*11]. GpVI was identified as a collagen receptor in a few Japanese patients with a mild bleeding problem whose platelets proved refractory to collagen[56,57,59,61]. An autoantibody against a 62 kDa protein lacking in a patient's platelets, aggregates heterologous platelets[120]. GpVI has two Ig folds, each stabilized by a single disulfide bond. Heavy glycosylation of a mucin-like region adjacent to the transmembrane domain is proposed to account for the apparent 62–65 kDa mass of the receptor on SDS–PAGE[114]. In megakaryoblastic cells, three alternative splice variants of GpVI have been described, two of which bind CRP and associate with Fc-receptor γ-chain[118]. GpVI-2 lacks 18 amino acids from the mucin-rich stem, whereas GpVI-3 lacks a functional transmembrane domain. These variant forms were not found in platelets. The cytoplasmic domain of GpVI contains a proline-rich region reminiscent of SH3 domain-binding motifs, suggesting a possible mode of signal transduction.

GpVI expression level is currently unknown, although convulxin, a snake venom protein from *Crotalus durissus terrificus* and a known ligand of GpVI, has around 300 high-affinity binding sites in platelets[121]. A subsequent study suggested about 1200 high-affinity sites for convulxin, about 15% of which could be inhibited by a synthetic triple-helical peptide, known as collagen-related peptide or CRP[122]. Crosslinked CRP contains repeating GPO motifs and is a potent GpVI-specific agonist[25,123] which has proved valuable in elucidating signalling (see pp. 165–167). Another 'mini-collagen', a triple-helical peptide containing a proposed $\alpha 2\beta 1$-recognition sequence from collagen IV, also contained eight repeating GPO motifs[24].

GpVI is currently considered to be the main collagen signalling receptor[124], mediating collagen-induced platelet activation and aggregation[125,*12,*13]. Stationary adhesion experiments, demonstrating cation-independent attachment to fibrillar collagen, probably reflect adhesion to GpVI[18,126]. However, in contrast to stationary adhesion[25], the use of CRP under conditions of blood flow showed that GpVI was incapable of mediating platelet attachment even at low shear rates[127]. A study using citrate-anticoagulated GpVI-deficient blood showed that biphasic platelet deposition was impaired at the level of aggregate formation, but not initial surface coverage[128]. Thus, it seems that receptors such as $\alpha 2\beta 1$ or TIIICBP are responsible for the initial platelet adhesion, and other receptors, such as GpVI, subsequently induce activation (see pp. 165–167), summarized currently as the two-site, two-step model of platelet–collagen interaction.

CD36 (GpIV, GpIIIb)

CD36 is a highly expressed receptor for both collagen and thrombospondin in platelets[129]. Its specificity for collagen over thrombospondin depends on phosphorylation of amino acids within residues 87–99 of its ectodomain[130]. CD36 is a scavenger receptor in macrophages, binding fatty acids and oxidized low-density lipoproteins, so having a possible role in atherogenesis, and in endothelial cells it binds malaria-infected red cells. The single isoform of CD36 was recently proposed to have two transmembrane domains with short cytoplasmic tails[131] (although this is subject to debate). CD36 is thought to associate with src-family tyrosine kinases[132] and with αIIbβ3[133]. Crosslinking of CD36 using antibodies causes platelet aggregation[134], dependent on FcγRIIa. The role of CD36 as a platelet collagen receptor is controversial, since most studies show that CD36-deficient platelets (Nak^a – phenotype, present in 3% of the Japanese population) support normal collagen-induced aggregation[135,136], adhesion

Cambridge Books Online © Cambridge University Press, 2009

under both static and flow conditions[35,137] and finally, have undisturbed signal transduction[138]. However, some studies revealed impaired platelet aggregation and adhesion to collagen type V[35,136], and impaired response to collagen type I[139,140]. These results were corroborated using polyclonal and monoclonal antibodies against CD36, demonstrating a role for CD36 in the early (2–5 min) phases of platelet-collagen interaction in flowing blood in the absence of Mg^{2+}, using native type I collagen fibrils as a substrate[141–143].

Other collagen receptors

65, 68/72 (TIIICBP) and 47 kDa receptors

Most of the findings concerning other collagen receptors in platelets[5] remain as single unconfirmed observations. However, analysis of two of these candidates has been carried further.

A collagen III-specific receptor was originally recognized using a synthetic linear octapeptide of sequence KPGEPGPK[144]. This peptide associated with two different proteins, 68 and 72 kDa, in ligand blotting after SDS–PAGE. A related peptide, KOGEOGPK, inhibited both platelet interaction with type III collagen or subendothelium under static and flow conditions[145] and activation of αIIbβ3[144]. Recently, the same peptide was used as an affinity ligand for p72, (TIIICBP) which has not yet been sequenced[*14]. The peptides described are linear, and when assembled in triple-helical conformation they did not support platelet adhesion under flow or static conditions[146].

The collagen I-specific 65 kDa receptor was first isolated using affinity chromatography with the single stranded α-chain from chick-skin type I collagen[147]. Oligonucleotide probes were designed from the CNBr-fragment sequence of the purified receptor. cDNA of p65 was cloned and sequenced from a human bone marrow library, and it lacks homology with any known receptor[148]. The 65 kDa receptor co-precipitated with the serine/threonine protein phosphatase 1, and was able to mediate phosphoinositide hydrolysis and calcium mobilisation[5].

Another collagen type III-specific platelet receptor, p47, was isolated using affinity chromatography as for the p65 protein, confirming that different collagens possess specific platelet recognition motifs[149]. However, the relationship of these two receptors with the other established receptors, as well as their place in the sequential adhesion of platelets to collagen, is not yet known.

p85

Like $\alpha2\beta1$ and GpVI, an 85 kDa protein was identified as a possible collagen receptor in platelets from a patient with a bleeding disorder and impaired responses to collagen. p85 could be discriminated from CD36 using mAbs, though not by electrophoresis[150]. No further data has been produced on p85.

cC1q-receptor

A complement receptor, cC1q-R, expressed at 4000 copies per platelet, has been suggested to be a collagen receptor[151]. Its defined ligand is the collagen-like, triple-helical tail of C1q[152]. cC1q-R is now accepted as a form of calreticulin[153], associating with the cell-surface through either a transmembrane domain or a KDEL sequence. Polymeric C1q has been reported to cause platelet aggregation, P-selectin expression and procoagulant activity[154]. Both C1q and anti-cC1q-R antibodies inhibited collagen-induced platelet aggregation, but not adhesion of platelets to collagen. cC1q-R remains to be confirmed as either a true collagen receptor, or as a modulator of other associated receptors.

Affinity regulation of receptors and accessory molecules

The affinity of $\alpha2\beta1$ and specificity of CD36 have been shown to alter after platelet activation. Several associated proteins either mediate inside-out signalling or co-operate with $\alpha2\beta1$. For GpVI, in contrast, a requirement only for the Fc receptor γ-chain has been demonstrated. This section addresses affinity regulation and the role of possible accessory proteins both outside and within the platelet.

Affinity regulation of $\alpha2\beta1$

The conformational shift (see Fig. 11.2) in $\alpha2\beta1$ integrin I-domain revealed upon ligand binding[102] may correlate with alteration in $\alpha2\beta1$ affinity which occurs with activating mAbs and divalent cations such as Mn^{2+}[87]. On the other hand, inside-out signalling has long been known to cause conformational changes and to promote soluble fibrinogen binding by αIIbβ3 (see Chapters 4 and 22)[155]. Such changes expose new binding sites, ligand- and receptor-induced binding sites (LIBS and RIBS) on αIIbβ3. For CD36, the dephosphorylation of its extracellular domain is claimed to switch ligand-specificity from collagen to thrombospondin[130].

Recently, it was suggested that the affinity of $\alpha2\beta1$ for collagen is also controlled by inside-out signalling[71,156,*15].

Cambridge Books Online © Cambridge University Press, 2009

Platelets do not bind soluble monomeric collagen, which must first be polymerised into fibrils. Pre-activation of platelets with ADP, thrombin, CRP or certain mAbs facilitated binding of soluble collagen[71,157], generating two states of affinity depending on the activating agonist, which was blocked by PGI_2[157] or inhibitors of intracellular signalling pathways[156]. Low-affinity ligand-binding was induced by ADP regardless of concentration, whereas thrombin or CRP could generate high-affinity interaction of soluble collagen with $\alpha 2\beta 1$. These results fit the observations of activation-dependent binding of platelets to collagen via $\alpha 2\beta 1$ under flow[158], in which an activatory mAb for $\alpha 2\beta 1$ was shown to slow platelets down, while inhibitory mAbs increased the velocity of the platelets over a collagen surface. Changes in binding affinity may determine substrate selectivity of $\alpha 2\beta 1$[86]. Although currently the regulation of affinity modulation is uncharacterized, the $\alpha 2$-domain has been shown to exert negative regulation[159], and the NPXY motif in the $\beta 1$-cytoplasmic domain is thought to be critical for inside-out signalling[160]. Finally, there is increasing evidence for associated intracellular molecules that may mediate these conformational changes, whereas the cysteine-rich protein disulfide isomerase-like domain in the $\beta 1$-subunit may regulate covalent changes in conformations from the outside (see p. 164, next section).

Protein disulphide isomerase

On activation, platelets express protein disulphide isomerase (PDI) activity on their surface[161] and into the external medium[162]. PDI, a 62 kDa protein, is normally considered an ER enzyme and chaperone, responsible for correct disulfide bonding and folding of nascent proteins. In this context, it also serves as the β-subunit of 4-prolylhydroxylase, binding collagen monomers to allow their hydroxylation[163]. PDI is not known to bind mature collagen, however.

PDI expressed on the platelet surface, perhaps secured by its KDEL sequence, appears to regulate the interaction of platelet glycoproteins and their ligands, and in vitro evidence suggests that it may also interact with other accessory molecules, such as calreticulin/cC1q-R[164]. PDI activity has been implicated in the function of GpIbα[165], in the association of thrombospondin with PDGF secreted from platelet α-granules[166], and in the regulation of the fibrinogen receptor, αIIb$\beta 3$, where it is believed to mediate reorganization of the disulfide bridges in the Cys-rich stem of the integrin β-subunit[167,168]. This region has marked sequence homology with the active site of PDI, suggesting that intrinsic PDI activity, and thiol re-arrangement, may be a general property of integrin β-subunits[169,170].

Thiol re-arrangement[171] and cell-surface PDI[172] have been shown to be important in the $\alpha 2\beta 1$-mediated interaction of platelets with collagen. Use of receptor-specific triple-helical peptides allows $\alpha 2\beta 1$ to be discriminated from GpVI in this context, showing the role of PDI to be integrin-specific (authors' unpublished data). No evidence exists thus far for a role for Ca^{2+} in regulating PDI activity, as may be the case in the ER. These data suggest that conversion between the high and low affinity states of $\alpha 2\beta 1$ could be catalysed by PDI activity.

Integrin-associated protein (CD47)

As its name implies, IAP associates with both αIIb$\beta 3$ and $\alpha 2\beta 1$ on the platelet surface[173,174]. This 52 kDa protein has a single Ig-like fold and five transmembrane domains. CD47 binds thrombospondin, and one model proposes that it co-operates with an integrin to deliver a signal to the cell interior, for example, by acting as an ion channel. A specific sequence (known as 4N1K) from thrombospondin binds CD47, inhibiting adenylate cyclase through G_i[175]. 4N1K has been reported to exhibit synergism with soluble collagen in activating platelets, an effect absent in CD47 knockout mice[174].

Fc receptors

Platelets express both the low-affinity IgG receptor, FcγRIIA, and the Fc-receptor γ-chain. Whilst signalling caused by crosslinking FcγRIIA has much in common with collagen-induced signalling[176,177], there is little evidence for direct involvement of FcγRIIA. However, FcγRIIA has been found to associate with GpIb/IX/V[178], and so could possibly mediate indirect signals from collagen through the vWf/collagen axis.

The structurally-distinct FcR γ-chain, in marked contrast, is essential for signal transduction from GpVI by collagen or CRP[179]. The association of GpVI with FcR γ-chain, occurring through an arginine–aspartate salt bridge within the plasma membrane[114] in resting and stimulated platelets[180–182], is essential for its expression[117,119]. FcR γ-chain is thought to represent the key internal apparatus for transmitting signals onwards from GpVI to the platelet interior, although it also associates with GpIb/IX/V[183]. FcR γ-chain is a disulfide-bridged dimer, with short external domains. Its cytoplasmic tail (42 amino acids) contains an immunoreceptor tyrosine activation motif (ITAM), a site which on phosphorylation recruits signalling proteins.

Cambridge Books Online © Cambridge University Press, 2009

Calreticulin

Intracellular forms of calreticulin may interact with a consensus sequence, KXGFFKR, in the integrin α-subunit cytoplasmic tail[184]. Calreticulin interacts with both αIIbβ3 in platelets and with $\alpha 2\beta 1$ in other cells, regulating their function and signalling[185–187]. The high-affinity form of $\alpha 2\beta 1$ was shown to interact with calreticulin in phorbol ester-treated Jurkat cells, where anticalreticulin antibodies reversed cell adhesion to collagen I[188].

PECAM-1 (CD31)

The platelet and endothelial cell adhesion molecule, PECAM-1, a member of the Ig superfamily, can bind and activate protein tyrosine phosphatases through its immunoreceptor tyrosine inhibitory motif (ITIM)[189,*17,*18,*19]. Platelet PECAM-1 is phosphorylated on tyrosine residues by c-src family kinases, and co-immunoprecipitates with them after activation by several ligands including collagen[190]. Recently, PECAM-1 was reported to inhibit the response of platelets to collagen[191,*20,*21]. In other cells, PECAM-1 oligomerisation induces integrin-mediated spreading[192].

CD9

The tetraspanin, CD9, associates with $\alpha 2\beta 1$ and other $\beta 1$ integrins on vascular smooth muscle cells, where it is believed to modulate collagen matrix reorganisation[193]. CD9 is expressed in platelets, where it complexes with glycoproteins, including αIIbβ3 and CD36[194–196]. Anti-CD9 antibodies stimulate platelet tyrosine kinase activity[197].

Venoms, etc.

The evolution of specific inhibitory or activatory proteins in snake venoms or the saliva of leeches and blood-sucking insects underscores the importance of the platelet-collagen interaction in haemostasis. These proteins may target either the receptors on platelets or collagen itself. Typically, they belong to either of two categories, which characterise their mode of action: the C-type lectin family, or the metalloproteinase-disintegrins. However, as these venoms often contain mixtures of active proteins or because the proteins have dual effects, experimental results may be contradictory and should be interpreted with care. These natural modulators may help us to understand the relevance of individual receptors and, crucially, may indicate important antithrombotic targets. We will mention briefly a few examples of functional significance[198–200].

As there is no readily available monoclonal antibody targeting GpVI, the multimeric protein, convulxin, from *Crotalus durissus terrificus,* has been used, like the CRP peptide, to specifically activate platelets. Many of the platelet activation events induced by very low convulxin concentrations, i.e. aggregation, Ca^{2+} signalling and tyrosine phosphorylation[201], resemble closely those induced by collagen fibres. Convulxin competes with CRP for one of its two binding sites on platelets[122] and although it has also been suspected to bind $\alpha 2\beta 1$[121], no receptor other than GpVI has been proven.

Although jararhagin and jaracetin from *Bothrops jararaca* were originally found to activate the vWf A1-domain, promoting vWf binding to GpIb/IX/V, because they blocked $\alpha 2\beta 1$-collagen binding through both the disintegrin and metalloproteinase domains, they were soon implemented in studying the platelet-collagen interaction[202]. Proteolysis of the $\beta 1$-subunit of the integrin was shown to mediate this inhibitory effect, together with direct binding to the $\alpha 2$-subunit[203,204]. A positively charged cyclic RKK peptide, based on the metalloproteinase domain, bound strongly to acidic amino acid sidechains near the $\alpha 2$–I domain MIDAS and inhibited collagen binding[205,206].

Other snake venom proteins claimed to be $\alpha 2\beta 1$-specific include alternagin (*Bothrops alternatus*)[207], trimucytin (*Trimeresurus mucrosquamatus*)[208] and rhodocytin/aggretin (*Calloselasma rhodostoma*)[209,210], and the two latter induce platelet aggregation. However, trimucytin was recently suggested to work through GpVI[211]. Interestingly, the use of rhodocytin supported the concept that $\alpha 2\beta 1$ can signal in platelets[212]. However, the specificity of these venom proteins for $\alpha 2\beta 1$ has been questioned. Neither soluble recombinant $\alpha 2\beta 1$ nor the wild-type integrin bound to rhodocytin. Instead, within the same venom, a 27 kDa antagonist of collagen-induced aggregation was identified as rhodocetin, a specific ligand for $\alpha 2\beta 1$[213,214]. The ability of proteins (pallidipin, moubatin and Tick Adhesion Inhibitor) from different species to perturb platelet–collagen interaction has been discussed elsewhere[1].

Finally, some venom proteins bind directly to collagen and thus hamper its interaction with either platelet $\alpha 2$ I-domain or vWf A-domain. Such proteins include catrocollastatin (*Crotalus atrox*)[215], LAPP (leech antiplatelet protein) and calin (*Hirudo medicinalis*)[216].

Signalling through collagen receptors

Collagen, like thrombin, is a strong platelet agonist, causing several separate activation events (arachidonate

Cambridge Books Online © Cambridge University Press, 2009

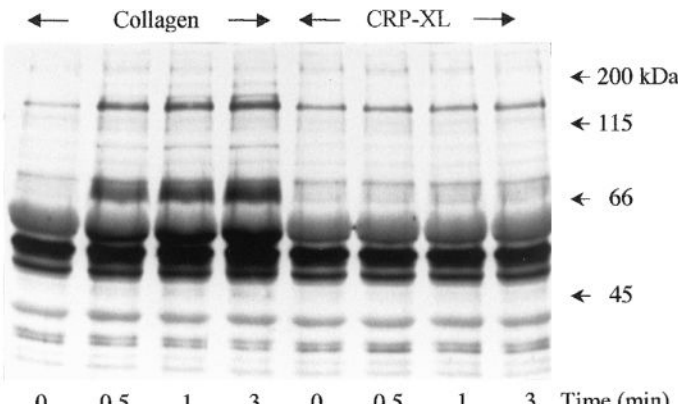


Fig. 11.3. Shows the response of GpVI-deficient platelets in suspension to collagen fibres and to CRP, over the indicated times. While CRP has no discernible effect on protein tyrosine phosphorylation (detected in Western blots), collagen caused a marked increase in protein phosphotyrosine, although it did not aggregate platelets. Thus, GpVI is both sufficient and necessary for full platelet aggregation, but collagen-induced signals are also mediated by other receptors. (Courtesy of T. Ichinohe and M. Okuma, Kyoto; collaborative data with the authors.)

release, morphological changes, secretion, aggregation and procoagulant activity). Pathways as diverse as G-protein linked signalling and tyrosine kinase activity converge upon intracellular calcium (Chapter 17) to elicit each of these defined signalling endpoints, which involve the interplay of a profusion of signalling molecules including cyclic nucleotides (Chapter 20), ADP (Chapter 9), H_20_2[217] and eicosanoids such as PGI_2 and TxA_2 (Chapter 15).

Immobilized collagens, in the context of the blood vessel wall, provide unique means for activation when compared with soluble ligands. They offer to the platelet a surface to spread upon, and by virtue of the highly organized structure of the collagen fibre, they present a repetitive array of receptor-recognition motifs. Thus, several novel concepts arise: receptor clustering may cause signals to propagate simply from the increased local concentration of receptors and their associated signalling proteins at the inner surface of the platelet plasma membrane. This increased receptor avidity has long been associated with integrin function in other cells. Further, the structure of collagen may support spatial and temporal association of dissimilar receptor populations, for example $\alpha 2\beta 1$ with GpVI, or either of these with CD36. The different protein species associated with the intracellular domains of these various receptors will support great diversity of signalling from collagen, but renders the dissection of particular signalling pathways highly complex.

The use of specific ligands, CRP and convulxin, demonstrates that GpVI ligation and/or clustering is sufficient for platelet activation. Early studies involving GpVI-deficient platelets indicate its essential role in platelet aggregation by collagen[56,57]. Some signals, however, as shown by protein tyrosine phosphorylation (see Fig. 11.3) including the specific activation of the intracellular kinase c-src[218] and the up-regulation of αIIbβ3 for fibrinogen binding[124], can arise through other collagen receptors, whose identity is hotly debated. The collagen fibre offers binding motifs to such receptors, and it is increasingly accepted that these contribute, especially under physiological blood flow[143], to the platelet deposition and activation process[219,220]. A key task remaining is to determine the degree of overlap of these two functions, and which other receptors participate.

Second messenger pathways stimulated by collagen

The cytoplasmic domains of platelet collagen receptors identified to date lack catalytic activity. Platelet activation therefore depends upon associated signalling molecules, especially tyrosine kinases, leading to the activation of phospholipase Cγ2[221,222]. This pathway results in the activation of protein kinase C together with elevation of cytosolic calcium (see Chapter 17), which is central to the platelet activation process.

Platelet activation involves controlled modulation of cyclic nucleotide levels (Chapter 20). Early work showed that collagen lowered cyclic AMP levels in platelets[223], a process blocked by pertussis toxin treatment[224], implicating G_i function[225]. Inhibition of adenylate cyclase by type I collagen fibres was independent of ADP or TxA_2 production[223,226], and the GpVI-specific CRP was inactive[21]. The peptide KPGEPGPK, from collagen III, has been implicated in cyclic AMP metabolism, suggesting a role for the TIIICBP[148], but the receptors responsible have not been formally identified. Collagen also stimulates guanylate cyclase activity, and cyclic GMP, like cyclic AMP, is an inhibitor of platelet activation (see Chapter 20). This paradoxical action may reside in the capacity of collagen to stimulate αIIbβ3-independent NO synthesis[227], which in turn stimulates soluble guanylate cyclase. It might thus represent a termination signal, restricting the extent of collagen-stimulated platelet activation and deposition at sites of vascular damage. Recently, the p65 type I collagen receptor was implicated in NO production[228].

GpVI signalling

Truncation of the cytosolic tail of GpVI in a rat leukemia cell line abrogated convulxin-stimulated signalling but not

Cambridge Books Online © Cambridge University Press, 2009

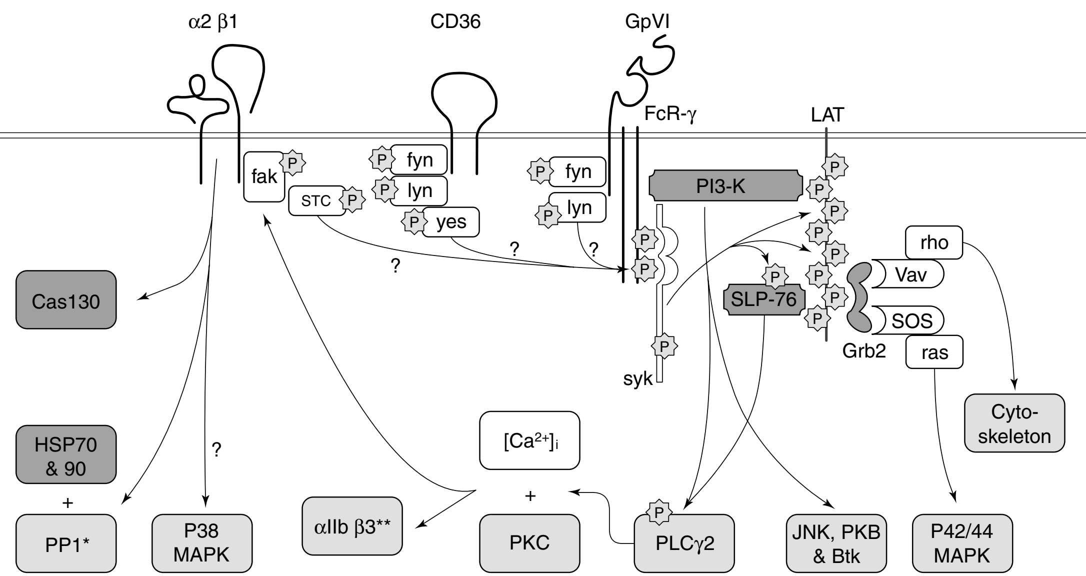


Fig. 11.4 (see also colour plate). Depicts proposed signalling pathways from the major collagen receptors, $\alpha 2\beta 1$, CD36 and GpVI. Phosphorylation events are shown using blue arrows, and other signalling pathways in black. Question marks indicate possible signalling pathways, lacking specific detail at present. Signalling molecules are defined in the text. Tyrosine kinases are coded *beige*, serine/threonine kinases *pink*, adapter proteins in *orange*. Tyrosine phosphorylation is indicated by stars on the relevant proteins. Other collagen receptors and possible signals arising from the GpIb/IX/V axis are omitted for clarity.

expression of GpVI, which depends on its association with the FcR γ-chain[119]. During the activation process, the ITAM motif of FcR γ becomes phosphorylated[181,*19], an event required for only one of the FcR γ-chains[180]. The GpVI/FcR γ-chain-associated src-family kinases fyn or lyn[229] have been proposed to mediate this event in both human[230] and mouse platelets[231].

A crucial question, yet to be answered, is how the activation of these kinases occurs, to allow phosphorylation of FcR γ-chain. The polyproline motif in the cytosolic tail of GpVI may bind these kinases through their SH3 domains. Other less direct possibilities exist, as fyn and lyn also associate with CD36[132], which might be engaged together with GpVI during the early phases of interaction with the collagen fibre[142,219]. However, since CD36-deficient platelets respond fully to collagen, $\alpha 2\beta 1$ is a more plausible candidate for a coreceptor: a recent report suggested that rhodocytin promotes the dissociation from $\alpha 2\beta 1$ and activation of c-src, and its subsequent binding and phosphorylation of p130cas[104].

Knowledge of platelet activation downstream from GpVI has developed rapidly, modelled on signalling pathways in T-cells[125]. The hematopoetic cell-specific tyrosine kinase, p72syk (spleen tyrosine kinase), is activated in response to collagen[218,232,233], and is essential for collagen- and CRP-induced signal transduction[179]. The phosphorylated ITAM of FcR γ-chain recruits p72syk, which is believed to phosphorylate the scaffold protein, LAT (linker for activation of T-cells), a prominent substrate for tyrosine kinases in collagen- and especially CRP-stimulated platelets, and SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa)[234,*22]. Signalling complexes can assemble on SLP-76 and LAT[235], so relaying signals to different apparatus including protein tyrosine phosphatases[236] within the platelet. Thus, PI-3-kinase (phosphatidylinositol 3-kinase) becomes recruited to the cell membrane by association with both LAT and FcR-γ chain[237], where it generates 3-phosphoinositides which may lead to the activation of PKB/Akt[238,239]. PI-3-kinase[240] and SLP-76 acting in concert[241] lead to PLC $\gamma 2$ becoming phosphorylated and activated, causing phosphoinositide turnover, Ca^{2+} signalling and PKC (protein kinase C) activation. Btk (Bruton's tyrosine kinase) and its homologue tec, activated by collagen[242], have also been implicated in mediating PLC $\gamma 2$ activation[243]. Guanine nucleotide exchange factors such as Vav1 and Sos (son of sevenless), recruited to signalling complexes via the adapter protein, Grb2 (growth factor receptor-bound2), activate small GTP-binding proteins and so initiate cytoskeletal change[244,245] or activate MAP kinases (mitogen-activated protein kinases). The role of adaptor proteins has been reviewed recently[246]. Fig. 11.4, see colour plate, summarizes the present incomplete understanding of these events.

Cambridge Books Online © Cambridge University Press, 2009

$\alpha 2\beta 1$ signalling

Although $\alpha 2\beta 1$ is an essential signalling receptor in other cells[247], regulating, for example, smooth muscle cell phenotype[248], signalling by $\alpha 2\beta 1$ in platelets is debated. The two-site two-step model implies tight binding of $\alpha 2\beta 1$ to collagen as a pre-requisite for the weaker interaction with GpVI. Thus, blockade of $\alpha 2\beta 1$ may indirectly inhibit signals from GpVI (or other receptors). On the other hand, $\alpha 2\beta 1$-generated signals may be masked by those from GpVI. Ligands that bind or crosslink $\alpha 2\beta 1$ cause neither aggregation of platelets in suspension nor discernible protein tyrosine phosphorylation[123], although blockade of $\alpha 2\beta 1$ by specific mAbs attenuated signalling from collagen[249]. Signals from $\alpha 2\beta 1$ appear to be more subtle than those from GpVI. However, platelet adhesion through $\alpha 2\beta 1$ to collagenous substrates induced phosphorylation of the focal adhesion kinase, p125fak, under either static or flow conditions[250,251], but a cross-linked triple-helical peptide specific for $\alpha 2\beta 1$ caused no discernible tyrosine phosphorylation of p125fak when applied to platelets in suspension[252]. It is plausible therefore that signals attributed to $\alpha 2\beta 1$ derive from the cytoskeletal re-organisation supported by immobilised ligands. Thus, the adhesive process itself amplified responses to GpVI ligands, although the basis for this remains to be resolved.

In two further studies assessing $\alpha 2\beta 1$ signalling in adherent platelets by TxA_2 generation and $\alpha IIb\beta 3$ activation, Mg^{2+}-dependent adhesion to monomeric collagen induced PAC-1 binding, but not TxA_2 generation[219,220]. Interestingly, TxA_2 generation stimulated by fibrillar collagen in the presence of Mg^{2+} was 3–4 times lower than in its absence, perhaps implying an inhibitory role for $\alpha 2\beta 1$.

Previously, phosphatase inhibitors have been shown to influence various aspects of collagen-induced platelet activation, from spreading to $\alpha IIb\beta 3$ upregulation. Under flow conditions, $\alpha 2\beta 1$ occupancy has been implicated in the activation of the serine/threonine phosphatase, PP1, which dissociates from its complex with heat shock protein (hsp) 90 and hsp70, an event correlating with dephosphorylation of several platelet proteins[253]. There is further evidence for phosphatase association with $\beta 1$ from other cells: PP2A associated with $\beta 1$ integrins in stem cells[254], and okadaic acid inhibited adhesion to collagen through $\alpha 2\beta 1$ in Jurkat cells[186].

The collagen-induced procoagulant response[255] is regulated by a balance between phosphorylation and dephosphorylation[256,257]. GpVI was shown to be an essential receptor[258], although incapable of eliciting procoagulant activity from platelets in suspension[259]. In contrast, the response was enhanced and partly dependent on $\alpha 2\beta 1$ in platelets adherent to collagen. Furthermore, inhibition of p38 MAP kinase reduced platelet blebbing, an event leading to procoagulant microvesiculation[259]. Previous work has shown a specific role for p38 MAP kinase in collagen-induced aggregation[260], and in other cells, p38 is thought to be activated by $\alpha 2\beta 1$ occupancy[261].

The limited evidence for direct signalling via $\alpha 2\beta 1$ may be explained if essential conformational changes in $\alpha 2\beta 1$ occur in adherent but not suspended platelets. $\alpha 2\beta 1$ signalling may also be dependent on accessory molecules. $\alpha 2\beta 1$ associates with co-receptors in other cell types, and in platelets $\alpha 2\beta 1$ has been suggested to co-exist with GpVI[262]. In platelets and other cells, $\beta 1$ integrins, CD36, GpIb/IX/V, activated lyn and uPAR also occur in cholesterol-rich membrane microdomains (although caveolin, often found in such structures, is absent)[263–265], which may prove to be sites for cooperative interaction between collagen receptors. Finally abnormal gangliosides from tumour cells or athero sclerotic plaques have been shown to enhance $\alpha 2\beta 1$ function[266,267].

Models for platelet–collagen interaction

Recent evidence has allowed the concept of the platelet–collagen interaction to develop from a two-site two-step process[52] into a multisite multistep model[8,145], implying that no single adhesion or activation receptor is responsible, but rather several receptors act in concert. This is reflected in the mild nature of bleeding disorders associated with single receptor deficiencies compared with those having several coinciding defects[63,268]. This also explains why collagen receptor polymorphisms may be difficult to associate with multifactorial diseases such as coronary thrombosis or stroke (see p. 160).

The detailed role of the various collagen receptors is beginning to emerge. Under high shear rate flow conditions, both the vWf–Ib/IX/V axis[269–271] and $\alpha 2\beta 1$ seem to be essential[78,143,272], although recent knock-out mouse data does not support this[*9]. The affinity of $\alpha 2\beta 1$ increases through adhesion-dependent platelet activation, and under those conditions, $\alpha 2\beta 1$ may elicit typical signalling reactions. GpVI, the major signalling receptor, unable to engage under flow conditions itself[146], may induce the high-affinity state of $\alpha 2\beta 1$[156]. CD36 is involved in very early platelet adhesion, but other receptors can compensate for its absence. Recently, TIIICBP was also shown to interact under high shear rate conditions as an early contact receptor with signalling capacity[145], and a similar pre-$\alpha 2\beta 1$-binding receptor was proposed for collagen type I[273].

The model in Fig. 11.5 suggests that as the platelet slows after vWf-Ib engagement, initial direct contact with colla-

Cambridge Books Online © Cambridge University Press, 2009

gen is by receptors such as TIIICBP or $\alpha2\beta1$, which are responsible for arrest of the platelet on the sub-endothelial collagen surface. This allows GpVI to be cross-linked to an extent that will support full-blown platelet activation and may also activate src-family kinases important for signal transmission through the GpVI-FcR γ-chain pathway. The resulting signals, or possibly even those from contact receptors, also transform $\alpha2\beta1$ into a high-affinity conformation. This final anchorage may then generate additional signal transduction, important for certain platelet responses such as procoagulant activity. The model does not take into account the various accessory co-receptors or molecules which may orchestrate and fine tune both platelet adhesion and activation.

Closing remarks

The complexity of platelet collagen receptor populations is matched by that of collagen, so to elucidate their interplay requires carefully defined experiment. Thus, flow studies using physiological cation concentrations and collagens in immobilized native form are required before a full understanding can emerge. Many discrepancies in the literature arise from the use of functionally dissimilar collagen preparations as platelet agonists.

The field is rapidly advancing. Since writing this chapter, novel collagen species have been described, and new studies on the comparative significance of receptors under flow have appeared. A better understanding is developing of collagen-induced signalling and the role of receptor polymorphisms in disease. Very recent references, marked in the text thus *, can be found at the end of the reference list.

References

1 Sixma JJ, van Zanten GH, Huizinga EG et al. Platelet adhesion to collagen: an update. *Thromb Haemost* 1997; 78: 434–438.
2 Moroi M, Jung SM. Platelet receptors for collagen. *Thromb Haemost* 1997; 78: 439–444.
3 Barnes MJ, Knight CG, Farndale RW. The collagen–platelet interaction. *Curr Opin Haematol* 1998; 5: 314–320.
4 Clemetson KJ. Platelet collagen receptors: a new target for inhibition? *Haemostasis* 1999; 29: 16–26.
5 Chiang TM. Collagen–platelet interaction: platelet non-integrin receptors. *Histol Histopathol* 1999; 14: 579–585.
6 Alberio L, Dale GL. Platelet–collagen interactions: membrane receptors and intracellular signalling pathways. *Eur J Clin Invest* 1999; 29: 1066–1077.
7 Barnes MJ, Farndale RW. Collagens and atherosclerosis. *Exp Gerontol* 1999; 34: 513–525.

Two-site, two-step model:

Collagen binding Signalling

Multi-site, multi-step model:

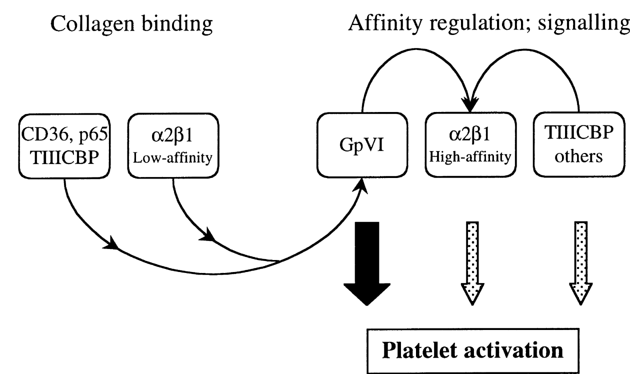


Fig. 11.5. Summarizes the two site, two-step multireceptor model together with the more intricate model of platelet activation that better fits current concepts of affinity regulation and signalling. The early role of GpIb/IX/V in adhesion and possibly signalling is omitted (see Chapters 12 and 21).

8 Watson S, Berlanga O, Best D, Frampton J. Update on collagen receptor interactions in platelets: is the two-state model still valid. *Platelets* 2000; 11: 252–258.
9 Hugues J. Agglutination précoce des plaquettes au cours de la formation du clou hémostatique. *Thromb Diath Haemorrh* 1959; 3: 177–186.
10 Bounameaux Y. L'accolement des plaquettes aux fibres sous-endothéliales. *Thromb Diath Haemorrh* 1961; 6: 504–516.
11 Hovig T. Aggregation of rabbit blood platelets produced in vitro by saline extract of tendons. *Thromb Diath Haemorrh* 1963; 9: 248–263.
12 Brass LF, Bensusan HB. The role of collagen quaternary structure in the platelet ratio: collagen interaction. *J Clin Invest* 1974; 54: 1480–1487.
13 Muggli R, Baumgartner HR. Collagen-induced platelet aggregation: requirement for tropocollagen multimers. *Thromb Res* 1973; 3: 715–728.
14 Jaffe R, Deykin D. Evidence for a structural requirement for the aggregation of platelets by collagen. *J Clin Invest* 1974; 53: 875–883.
15 Fitzsimmons CM, Barnes MJ. The platelet reactivity of the $\alpha2$(I)-chain of type I collagen: Platelet aggregation induced by polymers of the molecule $\alpha2$(I)]3. *Thromb Res* 1985; 39: 523–531.
16 Fitzsimmons CM, Cawston TE, Barnes MJ. The platelet reactivity of collagen Type I: evidence for multiple platelet-reactive

Cambridge Books Online © Cambridge University Press, 2009

sites in the Type I collagen molecule. *Thromb Haemost* 1986; 56: 95–99.
17 Morton LF, Fitzsimmons CM, Rauterberg J, Barnes MJ. Platelet reactive sites in collagen. *Biochem J* 1987; 248: 483–487.
18 Morton LF, Peachey AR, Barnes MJ. Platelet-reactive sites in collagens type I and type III. Evidence for separate adhesion and aggregatory sites. *Biochem J* 1989; 258: 157–163.
19 Saelman EUM, Morton LF, Barnes MJ et al. Platelet adhesion to cyanogen-bromide fragments of collagen $\alpha1$(I) under flow conditions. *Blood* 1993; 82: 3029–3033.
20 Fields CG, Grab B, Lauer JL, Miles AJ, Yu Y-C, Fields GB. Solid-phase synthesis of triple-helical collagen-model peptides. *Lett Peptide Sci* 1996; 3: 3–16.
21 Barnes MJ, Knight CG, Farndale RW. The use of collagen-based model peptides to investigate platelet-reactive sequences in collagen. *Biopolymers* 1996; 40: 383–397.
22 Fields GB, Prockop DJ. Perspectives on the synthesis and application of triple-helical, collagen-model peptides. *Biopolymers* 1996; 40: 345–357.
23 Morton LF, McCulloch IY, Barnes MJ. Platelet aggregation by a collagen-like synthetic peptide. *Thromb Res* 1993; 72: 367–372.
24 Rao GHR, Fields CG, White JG, Fields GB. Promotion of human platelet adhesion and aggregation by a synthetic, triple-helical 'mini-collagen'. *J Biol Chem* 1994; 269: 13899–13903.
25 Morton LF, Hargreaves PG, Farndale RW, Young RD, Barnes MJ. Integrin $\alpha2\beta1$-independent activation of platelets by simple collagen-like peptides: collagen tertiary (triple helical) structure and quaternary (polymeric) structure are sufficient alone for $\alpha2\beta1$-independent platelet reactivity. *Biochem J* 1995; 306: 337–344.
26 Knight CG, Morton LF, Onley DJ et al.Identification in collagen type I of an integrin $\alpha_2\beta_1$-binding site containing an essential GER sequence. *J Biol Chem* 1998; 273: 33287–33294.
27 Knight CG, Morton LF, Peachey AR, Tuckwell DS, Farndale RW, Barnes MJ. The collagen-binding A-domains of integrin $\alpha1\beta1$ and $\alpha2\beta1$ recognize the same specific amino acid sequence, GFOGER, in native (triple-helical) collagens. *J Biol Chem* 2000; 275: 35–40.
28 Xu Y, Gurusiddappa S, Rich RL et al. Multiple binding sites in collagen type I for the integrins $\alpha1\beta1$ and $\alpha2\beta1$. *J Biol Chem* 2000; 275: 38981–38989.
29 Kehrel B. Platelet–collagen interactions. *Semin Thromb Haemost* 1995; 21: 123–129.
30 Prockop DJ, Kivirikko KI. Collagens: molecular biology, diseases, and potentials for therapy. *Annu Rev Biochem* 1995; 64: 403–434.
31 Barnes MJ. Collagens in atherosclerosis. *Collagen Related Res* 1985; 5: 65–97.
32 van Zanten GH, de Graaf S, Slootweg PJ et al. Increased platelet deposition on atherosclerotic coronary arteries. *J Clin Invest* 1994; 93: 615–632.
33 Mayne R. Collagenous proteins of blood vessels. *Arteriosclerosis* 1986; 6: 585–593.
34 Barnes MJ. In Nimni ME, ed. *Collagen, Vol 1: Biochemistry,* CRC Press: Boca Raton, FL; 1988; 275–290.
35 Saelman EUM, Nieuwenhuis HK, Hese KM et al. Platelet adhesion to collagen types I through VIII under conditions of stasis and flow is mediated by GPIa/IIa ($\alpha2\beta1$-integrin). *Blood* 1994; 83: 1244–1250.
36 Alberio L, Dale GL. Flow cytometric analysis of platelet activation by different collagen types present in the vessel wall. *Br J Haematol* 1998; 102: 1212–1218.
37 Bella J, Eaton M, Brodsky B, Berman HM. Crystal and molecular structure of a collagen-like peptide at 1.9Å resolution. *Science* 1994; 266: 75–81.
38 Ramshaw JAM, Shah NK, Brodsky B. Gly-X-Y tripeptide frequencies in collagen: a context for host-guest triple-helical peptides. *J Struct Biol* 1998; 122: 86–91.
39 Prockop DJ, Kivirikko KI, Tuderman L, Guzman NA. The biosynthesis of collagen and its disorders (Pt 1). *N Engl J Med* 1979; 301: 13–22.
40 Persikov AV, Ramshaw JAM, Kirkpatrick A, Brodsky B. Amino acid propensities for the collagen triple-helix. *Biochemistry* 2000; 39: 14960–14967.
41 Colombatti A, Bonaldo P, Doliana R. Type A modules: interacting domains found in several non-fibrillar collagens and in other extracellular matrix proteins. *Matrix* 1993; 13: 297–306.
42 Miller EJ, Rhodes RK. Preparation and characterization of the different types of collagen. *Methods Enzymol* 1982; 82: 36–64.
43 Rubin AL, Drake MP, Davison PF, Pfahl D, Speakman PT, Schmitt FO. Effects of pepsin treatment on the interaction properties of tropocollagen macromolecules. *Biochemistry* 1965; 4: 181–195.
44 Drake MP, Davison PF, Bump S, Schmitt FO. Action of proteolytic enzymes on tropocollagen and insoluble collagen. *Biochemistry* 1966; 5: 301–312.
45 Leibovich SJ, Weiss JB. Electron microscope studies of the effects of endo- and exopeptidase digestion on tropocollagen: a novel concept of the role of terminal regions in fibrillogenesis. *Biochim Biophys Acta* 1970; 214: 445–454.
46 Helseth Jr DL, Veis A. Collagen self-assembly in vitro: differentiating specific telopeptide-dependent interactions using selective enzyme modification and the addititon of free amino telopeptide. *J Biol Chem* 1981; 256: 7118–7128.
47 Holmes DF, Capaldi MJ, Chapman JA. Reconstitution of collagen fibrils in vitro; the assembly process depends on the initiating procedure. *Int J Biol Macromolecules* 1986; 8: 161–166.
48 Kadler KE, Holmes DF, Trotter JA, Chapman JA. Collagen fibril formation. *Biochem J* 1996; 316: 1–11.
49 Kuznetsova N, Leikin S. Does the triple helical domain of type I collagen encode molecular recognition and fiber assembly while telopeptides serve as catalytic domains? Effect of proteolytic cleavage on fibrillogenesis and on collagen–collagen interaction in fibers. *J Biol Chem* 1999; 274: 36083–36088.
50 Eyre DR. Collagen: molecular diversity in the body's protein scaffold. *Science* 1980; 207: 1315–1322.
51 Scott JE. Proteoglycan–fibrillar collagen interactions. *Biochem J* 1988; 252: 313–323.
52 Santoro SA, Walsh J, Staatz W, Baranski K. Distinct determinants

Cambridge Books Online © Cambridge University Press, 2009

on collagen support $\alpha 2\beta 1$ integrin-mediated platelet adhesion and platelet activation. *Cell Regulation* 1991; 2: 905–913.
53 Clemetson KJ. Primary haemostasis: sticky fingers cement the relationship. *Curr Biol* 1999; 9: R110–R112.
54 Nieuwenhuis HK, Akkerman JWN, Houdijk WPM, Sixma JJ. Human blood platelets showing no response to collagen fail to express surface glycoprotein Ia. *Nature* 1985; 318: 470–472.
55 Nieuwenhuis HK, Sakariassen K, Houdijk WPM, Nievelstein PFEM, Sixma JJ. Deficiency of platelet membrane glycoprotein Ia associated with a decreased platelet adhesion to subendothelium: a defect in platelet spreading. *Blood* 1986; 68: 692–695.
56 Moroi M, Jung SM, Okuma M, Shinmyozu K. A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion. *J Clin Invest* 1989; 84: 1440–1446.
57 Sugiyama T, Okuma M, Ushikubi F, Uchino H. A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and auto-immune thrombocytopenia. *Blood* 1987; 69: 1712–1720.
58 Deckmyn H, Chew SL, Vermylen J. Lack of platelet response to collagen associated with an autoantibody against glycoprotein Ia: a novel cause of acquired qualitative platelet dysfunction. *Thromb Haemost* 1990; 63: 74–79.
59 Ryo Y, Yoshida A, Sugano W et al. Deficiency of P62, a putative collagen receptor, in platelets from a patient with defective collagen-induced platelet aggregation. *Am J Hematol* 1992; 39: 25–31.
60 Arai M, Yamamoto N, Moroi M, Akamatsu N, Fukutake K, Tanoue K. Platelets with 10% of the normal amount of glycoprotein VI have an impaired response to collagen that results in a mild bleeding tendency. *Br J Haematol* 1995; 89: 124–130.
61 Ishibashi T, Ichinohe T, Sugiyama T, Takayama H, Titani K, Okuma M. Functional significance of platelet membrane glycoprotein p62 (GP VI), a putative collagen receptor. *Int J Hematol* 1995; 62: 107–115.
62 Handa M, Watanabe K, Kawai Y et al. Platelet unresponsiveness to collagen: involvement of glycoprotein Ia–IIa ($\alpha 2\beta 1$ integrin) deficiency associated with a myeloproliferative disorder. *Thromb Haemost* 1995; 73: 521–528.
63 Kehrel B, Balleisen L, Kokott R et al. Deficiency of intact thrombospondin and membrane glycoprotein Ia in platelets with defective collagen-induced aggregation and spontaneous loss of disorder. *Blood* 1988; 71: 1074–1078.
64 Siegel BM, Friedman IA, Schwartz SO. Hemorrhagic disease in osteogenesis imperfecta: Study on platelet functional defect. *Am J Med* 1957; 22: 315–322.
65 Johnson GJ, Holloway DE, Hutton SW, Duane WC. Platelet function in scurvy and experimental human vitamin C deficiency. *Thromb Res* 1981; 24: 85–96.
66 Hindriks GA, Sixma JJ, de Groot PG. Ascorbic acid increases thrombogenicity of cellular matrices. *Thromb Haemost* 1991; 66: 505–509.
67 Badimon JJ, Zaman A, Helft G, Fayad Z, Fuster V. Acute coronary syndromes: pathophysiology and preventive priorities. *Thromb Haemost* 1999; 82: 997–1004.
68 Maresta A, Balducelli M, Cantini L et al. Trapidil (triazolopyrimidine), a platelet-derived growth factor antagonist, reduces restenosis after percutaneous transluminal coronary angioplasty: results of the randomized, double-blind STARC study. *Circulation* 1994; 90: 2710–2715.
69 Kritzik M, Savage B, Nugent DJ, Santoso S, Ruggeri ZM, Kunicki TJ. Nucleotide polymorphisms in the alpha 2 gene define multiple alleles that are associated with differences in platelet alpha 2 beta 1 density. *Blood* 1998; 92: 382–388.
70 Kunicki TJ, Orchekowski RP, Annis D, Honda Y. Variability of integrin $\alpha 2\beta 1$ activity on human platelets. *Blood* 1993; 82: 2693–2703.
71 Jung SM, Moroi M. Platelets interact with soluble and insoluble collagens through characteristically different reactions. *J Biol Chem* 1998; 273: 14827–14837.
72 Moshfegh K, Wuillemin WA, Redondo M et al. Association of two silent polymorphisms of the platelet glycoprotein Ia/IIa receptor associated with risk of myocardial infarction: a case-control study. *Lancet* 1999; 353: 351–354.
73 Carlsson LE, Santoso S, Spitzer C, Kessler C, Greinacher A. The $\alpha 2$ gene coding sequence T807/A873 of the platelet collagen receptor integrin $\alpha 2\beta 1$ might be a risk factor for the development of stroke in younger patients. *Blood* 1999; 93: 3583–3586.
74 Santoso S, Kunicki TJ, Kroll H, Haberbosch W, Gardemann A. Association of the platelet glycoprotein Ia $C_{807}T$ gene polymorphism with nonfatal myocardial infarction in younger patients. *Blood* 1999; 93: 2449–2453.
75 Reiner AP, Kumar PN, Schwartz SM et al. Genetic variants of platelet glycoprotein receptors and risk of stroke in young women. *Stroke* 2000; 31: 1628–1633.
76 Roest M, Banga JD, Grobbee DE et al. Homozygosity for 807 T polymorphism in alpha(2) subunit of platelet alpha(2)beta(1) is associated with increased risk of cardiovascular mortality in high-risk women. *Circulation* 2000; 102: 1645–50.
77 Santoro SA. Platelet surface collagen receptor polymorphisms: variable receptor expression and thrombotic/hemorrhagic risk. *J Am Soc Hematol* 1999; 93: 3575–3577.
78 Di Paola J, Federici AB, Mannucci PM et al. Low platelet $\alpha_2\beta_1$ levels in type I von Willebrand disease correlate with impaired platelet function in a high shear stress system. *Blood* 1999; 93: 3578–3582.
79 Anvari A, Janisiw M, Turel Z, Huber K, Fischer G, Panzer S. Platelet glycoprotein Ia gene dimorphism alpha2–807 in malignant arrhythmia in coronary artery disease. *Thromb Res* 2000; 98: 281–286.
80 Corral J, Gonzalez-Conejero R, Rivera J, Ortuno F, Aparicio P, Vicente V. Role of the 807 C/T polymorphism of the alpha2 gene in platelet GP Ia collagen receptor expression and function – effect in thromboembolic diseases. *Thromb Haemost* 1999; 81: 951–956.
81 von Beckerath N, Koch W, Mehilli J, Bottiger C, Schomig A, Kastrati A. Glycoprotein Ia gene C807T polymorphism and risk for major adverse cardiac events within the first 30 days after coronary artery stenting. *Blood* 2000; 95: 3297–3301.
82 Kroll H, Gardemann A, Fechter A, Haberbosch W, Santoso S.

Cambridge Books Online © Cambridge University Press, 2009

The impact of the glycoprotein Ia collagen receptor subunit A1648G gene polymorphism on coronary artery disease and acute myocardial infarction. *Thromb Haemost* 2000; 83: 392–396.

83 Santoso S, Amrhein J, Hofmann HA et al. A point mutation Thr(799)Met on the alpha(2) integrin leads to the formation of new human platelet alloantigen Sit(a) and affects collagen-induced aggregation. *Blood* 1999; 94: 4103–4111.

84 Nieswandt B, Schulte V, Bergmeier W et al. Long-term antithrombotic protection by in vivo depletion of platelet glycoprotein VI in mice. *J Exp Med* 2001; 193: 459–469.

85 Vogel W, Gish GD, Alves F, Pawson T. The discoidin domain receptor tyrosine kinases are activated by collagen. *Mol Cell* 1997; 1: 13–23.

86 Santoro SA, Zutter MM. The $\alpha 2\beta 1$ integrin: a collagen receptor on platelets and other cells. *Thromb Haemost* 1995; 74: 813–821.

87 Humphries MJ. Integrin structure. *Biochem Soc Trans* 2000; 28: 311–340.

88 Languino LR, Ruoslahti E. An alternative form of the integrin β_1 subunit with a variant cytoplasmic domain. *J Biol Chem* 1992; 267: 7116–7120.

89 Coller BS, Beer JH, Scudder LE, Steinberg MH. Collagen-platelet interactions: evidence for a direct interaction of collagen with platelet GPIa/IIa and an indirect interaction with platelet GPIIb/IIIa mediated by adhesive proteins. *Blood* 1989; 74: 182–192.

90 Bergelson JM, Shepley MP, Chan BMC, Hemler ME, Finberg RW. Identification of the integrin VLA-2 as a receptor for echovirus-1. *Science* 1992; 255: 1718–1720.

91 Kamata T, Puzon W, Takada Y. Identification of putative ligand binding sites within I domain of integrin $\alpha_2\beta_1$ (VLA-2, CD49b/CD29). *J Biol Chem* 1994; 269: 9659–9663.

92 Dickeson SK, Mathis NL, Rahman M, Bergelson JM, Santoro SA. Determinants of ligand binding specificity of the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins. *J Biol Chem* 1999; 274: 32182–32191.

93 Tuckwell DS, Smith L, Korda M et al. Monoclonal antibodies identify residues 199–216 of the integrin $\alpha 2$ vWFA domain as a functionally important region within $\alpha 2\beta 1$. *Biochem J* 2000; 350: 485–493.

94 Springer TA. Folding of the N-terminal, ligand-binding region of integrin α-subunits into a β-propeller domain. *Proc Natl Acad Sci USA* 1997; 94: 65–72.

95 Pulcinelli FM, Sebastiani S, Pesciotti M, Pignatelli P, Gazzaniga PP, Daniel JL. Nickel enhances collagen-induced platelet activation acting by increasing the organization of the cytoskeleton. *Thromb Haemost* 1998; 79: 395–399.

96 Onley DJ, Knight CG, Tuckwell DS, Barnes MJ, Farndale RW. Micromolar Ca^{2+} is essential for Mg^{2+} -dependent binding of collagen by the integrin $\alpha 2\beta 1$ in human platelets. *J Biol Chem* 2000; 275: 24560–24566.

97 Fujimura K, Phillips DR. Calcium cation regulation of glycoprotein IIb–IIIa complex formation in platelet plasma membranes. *J Biol Chem* 1983; 258: 10247–10252.

98 Phillips DR, Baughan AK. Fibrinogen binding to human platelet plasma membranes. Identification of two steps requiring divalent cations. *J Biol Chem* 1983; 258: 10240–10246.

99 Springer TA, Jing H, Takagi J. A novel Ca^{2+} binding β hairpin loop better resembles integrin sequence motifs than the EF hand. *Cell* 2000; 102: 275–277.

100 Dickeson SK, Walsh JJ, Santoro SA. Contributions of the I and EF hand domains to the divalent cation-dependent collagen binding activity of the $\alpha 2\beta 1$ integrin. *J Biol Chem* 1997; 272: 7661–7668.

101 Santoro SA. Identification of a 160000 dalton platelet membrane protein that mediates the initial divalent cation-dependent adhesion of platelets to collagen. *Cell* 1986; 46: 913–920.

102 Emsley J, Knight CG, Farndale RW, Barnes MJ, Liddington RC. Structural basis of collagen recognition by $\alpha 2\beta 1$. *Cell* 2000; 101: 47–56.

103 Kamata T, Takada Y. Direct binding of collagen to the I domain of integrin $\alpha_2\beta_1$ (VLA-2, CD49b/CD29) in a divalent cation-independent manner. *J Biol Chem* 1994; 269: 26006–26010.

104 Suzuki-Inoue K, Ozaki Y, Kainoh M et al. Rhodocytin induces platelet aggregation by interacting with glycoprotein Ia/IIa (GPIa/IIa, integrin $\alpha 2\beta 1$). *J Biol Chem* 2000; 276: 1643–1652.

105 Kamata T, Liddington RC, Takada Y. Interaction between collagen and the $\alpha 2$ I-domain of integrin $\alpha 2\beta 1$. *J Biol Chem* 1999; 274: 32108–32111.

106 Käpylä J, Ivaska J, Riikonen R et al. Integrin α_2I domain recognizes type I and type IV collagens by different mechanisms. *J Biol Chem* 2000; 275: 3348–3354.

107 Tuckwell DS, Reid KBM, Barnes MJ, Humphries MJ. Integrin $\alpha 2$ A-domain binds specifically to a range of collagens but is not a general receptor for the collagenous motif. *Eur J Biochem* 1996; 241: 732–739.

108 Bella J, Berman HM. Integrin-collagen complex: a metal-glutamate handshake. *Structure* 2000; 8: R121–R126.

109 Vandenberg P, Kern A, Ries A, Luckenbill-Edds L, Mann K, Kühn K. Characterization of a type IV collagen major cell binding site with affinity to the $\alpha 1\beta 1$ and the $\alpha 2\beta 1$ integrins. *J Cell Biol* 1991; 113: 1475–1483.

110 Staatz WD, Fok KF, Zutter MM, Adams SP, Rodriguez BA, Santoro SA. Identification of a tetrapeptide recognition sequence for the a2b1-binding sites in collagen I. *J Biol Chem* 1991; 266: 7363–7367.

111 Yamaguchi K, Ura H, Yasoshima T, Shishido T, Denno R, Hirata K. Establishment and characterization of a human gastric carcinoma cell line that is highly metastatic to lymph nodes. *J Exp Clin Cancer Res* 2000; 19: 113–120.

112 Mizuno M, Fujisawa R, Kuboki Y. Type I collagen-induced osteoblastic differentiation of bone-marrow cells mediated by collagen-alpha 2 beta 1 integrin interaction. *J Cell Physiol* 2000; 184: 207–213.

113 Werr J, Johansson J, Eriksson EE, Hedqvist P, Ruoslahti E, Lindbom L. Integrin $\alpha 2\beta 1$ (VLA-2) is a principal receptor used by neutrophils for locomotion in extravascular tissue. *Blood* 2000; 95: 1804–1809.

114 Clemetson JM, Polgar J, Magnenat E, Wells TNC, Clemetson KJ. The platelet collagen receptor glycoprotein VI is a member

Cambridge Books Online © Cambridge University Press, 2009

of the immunoglobulin superfamily closely related to Fc alpha R and the natural killer receptors. *J Biol Chem* 1999; 274: 29019–29024.
115 Jandrot-Perrus M, Busfield S, Lagrue A-H et al. Cloning, characterization, and functional studies of human and mouse glycoprotein VI: a platelet-specific collagen receptor from the immunoglobulin superfamily. *Blood* 2000; 96: 1798–1807.
116 Miura Y, Ohnuma M, Jung SM, Moroi M. Cloning and expression of the platelet-specific collagen receptor glycoprotein VI. *Thromb Res* 2000; 98: 301–309.
117 Nieswandt B, Bergmeier W, Schulte V, Rackebrandt K, Gessner JE, Zirngibl H. Expression and function of the mouse collagen receptor glycoprotein VI is strictly dependent on its association with the FcRγ chain. *J Biol Chem* 2000; 275: 23998–24002.
118 Ezumi Y, Uchiyama T, Takayama H. Molecular cloning, genomic structure, chromosomal localization, and alternative splice forms of the platelet collagen receptor glycoprotein VI. Biochem. *Biophys Res Commun* 2000; 277: 27–36.
119 Zheng Y-M, Liu C, Chen H, Locke D, Ryan JC, Kahn ML. Expression of the platelet receptor GPVI confers signalling via the Fc receptor γ chain in response to the snake venom convulxin but not to collagen. *J Biol Chem* 2001; 276: 12999–13006.
120 Sugiyama T, Ishibashi T, Okuma M. Functional role of the antigen recognized by an antiplatelet antibody specific for a putative collagen receptor in platelet–collagen interaction. *Int J Hematol* 1993; 58: 99–104.
121 Jandrot-Perrus M, Lagrue A-H, Okuma M, Bon C. Adhesion and activation of human platelets induced by convulxin involve glycoprotein VI and integrin $\alpha 2\beta 1$. *J Biol Chem* 1997; 272: 27035–27041.
122 Niedergang F, Alcover A, Knight CG et al. Convulxin binding to platelet receptor GPVI: Competition with collagen related peptides. *Biochem Biophys Res Commun* 2000; 273: 246–250.
123 Knight CG, Morton LF, Onley DJ et al. Collagen-platelet interaction: Gly-Pro-Hyp is uniquely specific for platelet GpVI and mediates platelet activation by collagen. *Cardiovasc Res* 1999; 41: 450–457.
124 Kehrel B, Wierwille S, Clemetson KJ et al. Glycoprotein VI is a major collagen receptor for platelet activation: it recognizes the platelet activating quaternary structure of collagen, whereas CD36, GPIIb/IIIa and VWf do not. *Blood* 1998; 91: 491–499.
125 Watson SP. Collagen receptor signalling in platelets and megakaryocytes. *Thromb Haemost* 1999; 82: 365–376.
126 Zijenah LS, Morton LF, Barnes MJ. Platelet adhesion to collagen. Factors affecting Mg^{2+}-dependent and bivalent cation-independent adhesion. *Biochem J* 1990; 268: 481–486.
127 Verkleij MW, Morton LF, Knight CG, de Groot PG, Barnes MJ, Sixma JJ. Simple collagen-like peptides support platelet adhesion under static but not under flow conditions: Interaction via alpha2 beta 1 and von Willebrand factor with specific sequences in native collagen is a requirement to resist shear forces. *Blood* 1998; 91: 3808–3816.
128 Moroi M, Jung SM, Shinmyozu K, Tomiyama Y, Ordinas A, Diaz-Ricart M. Analysis of platelet adhesion to a collagen-coated surface under flow conditions – the involvement of glycoprotein VI in the platelet adhesion. *Blood* 1996; 88: 2081–2092.
129 Daviet L, McGregor JL. Vascular biology of CD36: Roles of this new adhesion molecule family in different disease states. *Thromb Haemost* 1997; 78: 65–69.
130 Asch A, Liu I, Briccetti FM et al. Analysis of CD36 binding domains: ligand specificity controlled by dephosphorylation of an ectodomain. *Science* 1993; 262: 1436–1440.
131 Gruarin P, Thorne RF, Dorahy DJ, Burns GF, Sitia R, Alessio M. CD36 is a ditopic glycoprotein with the N-terminal domain implicated in intracellular transport. *Biochem Biophys Res Commun* 2000; 275: 446–454.
132 Huang M-M, Bolen JB, Barnwell JW, Shattil SJ, Brugge JS. Membrane glycoprotein IV (CD36) is physically associated with the *Fyn, Lyn* and *Yes* protein-tyrosine kinases in human platelets. *Proc Natl Acad Sci* (USA) 1991; 88: 7844–7848.
133 Dorahy DJ, Berndt MC, Shafren DR, Burns GF. CD36 is spatially associated with glycoprotein IIb–IIIa (αIIbβ3) on the surface of resting platelets. *Biochem Biophys Res Commun* 1996; 218: 575–581.
134 Aiken ML, Ginsberg MH, Byers-Ward V, Plow EF. Effects of OKM5, a monoclonal antibody to glycoprotein IV, on platelet aggregation and thrombospondin surface expression. *Blood* 1990; 76: 2501–2509.
135 Yamamoto N, Akamatsu N, Yamazaki H, Tanoue K. Normal aggregations of glycoprotein IV (CD36)-deficient platelets from seven healthy Japanese donors. *Br J Haematol* 1992; 81: 86–92.
136 Kehrel B, Kronenberg A, Rauterberg J et al. Platelets deficient in glycoprotein IIIb aggregate normally to collagens type I and III but not to collagen type V. *Blood* 1993; 82: 3364–3370.
137 McKeown L, Vail M, Williams S, Kramer W, Hansmann K, Gralnick H. Platelet adhesion to collagen in individuals lacking glycoprotein IV. *Blood* 1994; 83: 2866–2871.
138 Daniel JL, Dangelmaier C, Strouse R, Smith JB. Collagen induces normal signal transduction in platelets deficient in CD36 (platelet glycoprotein IV). *Thromb Haemost* 1994; 71: 353–356.
139 Tandon NN, Ockenhouse CF, Greco NJ, Jamieson GA. Adhesive functions of platelets lacking glycoprotein IV (CD36). *Blood* 1991; 78: 2809–2813.
140 Díaz-Ricart M, Tandon NN, Carretero M, Ordinas A, Bastida E, Jamieson GA. Platelets lacking functional CD36 (glycoprotein IV) show reduced adhesion to collagen in flowing blood. *Blood* 1993; 82: 491–496.
141 Matsuno K, Diaz-Ricart M, Montgomery RR, Aster RH, Jamieson GA, Tandon NN. Inhibition of platelet adhesion to collagen by monoclonal anti-CD36 antibodies. *Br J Haematol* 1996; 92: 960–967.
142 Díaz-Ricart M, Tandon NN, Gomez-Ortiz G et al. Antibodies to CD36 (GPIV) inhibit platelet adhesion to subendothelial surfaces under flow conditions. *Arterioscler Thrombos Vasc Biol* 1996; 16: 883–888.
143 Siljander P, Lassila R. Studies of adhesion-dependent platelet

Cambridge Books Online © Cambridge University Press, 2009

activation: distinct roles for different participating receptors can be dissociated by proteolysis of collagen. *Arterioscler Thromb Vasc Biol* 1999; 19: 3033–3048.
144 Karniguian A, Legrand YJ, Lefrancier P, Caen JP. Effect of a collagen-derived octapeptide on different steps of the platelet/collagen interaction. *Thromb Res* 1983; 32: 593–604.
145 Monnet E, Fauvel-Lafeve F. A new platelet receptor specific to Type III collagen. Type III collagen-binding protein. *J Biol Chem* 2000; 275: 10912–10917.
146 Verkleij MW, Ijsseldijk MJW, Heijnen-Snyder GJ et al. Adhesive domains in the collagenIII fragment $\alpha1$(III)CB4 that support $\alpha2\beta1$– and von Willebrand factor-mediated platelet adhesion under flow conditions. *Thromb Haemost* 1999; 82: 1137–1144.
147 Chiang TM, Kang AH. Isolation and purification of collagen $\alpha1$(I) receptor from human platelet membrane. *J Biol Chem* 1982; 257: 7581–7586.
148 Chiang TM, Rinaldy A, Kang AH. Cloning, characterization and functional studies of a non-integrin platelet receptor for type I collagen. *J Clin Invest* 1997; 100: 514–521.
149 Chiang TM, Seyer JM, Kang AH. Collagen-platelet interaction – separate receptor sites for types I and III collagen. *Thromb Res* 1993; 71: 443–456.
150 Deckmyn H, van Houtte E, Vermylen J. Disturbed platelet aggregation to collagen associated with an antibody against an 85–90 kD platelet glycoprotein in a patient with prolonged bleeding times. *Blood* 1992; 79: 1466–1471.
151 Peerschke EIB, Ghebrehiwet B. Platelet receptors for the complement component C1q: Implications for hemostasis and thrombosis. *Immunobiology* 1998; 199: 239–249.
152 Eggleton P, Reid KBM, Tenner AJ. C1q – how many functions? How many receptors? *Trends Cell Biol* 1998; 8: 428–431.
153 Sim RB, Moestrup SK, Stuart GR et al. Interaction of C1q and the collectins with the potential receptors calreticulin (cC1qR/Collectin Receptor) and megalin. *Immunobiology* 1998; 199: 208–224.
154 Peerschke EIB, Reid KBM, Ghebrehiwet B. Platelet activation by $C1_q$ results in the induction of αIIb/$\beta3$ integrins (GPIIb–IIIa) and the expression of P-selectin and procoagulant activity. *J Exp Med* 1993; 178: 579–587.
155 Shattil SJ, Kashiwagi H, Pampori N. Integrin signalling: the platelet paradigm. *Blood* 1998; 91: 2645–2657.
156 Jung SM, Moroi M. Signal-transducing mechanisms involved in activation of the platelet collagen receptor integrin $\alpha_2\beta_1$. *J Biol Chem* 2000; 275: 8016–8026.
157 Kainoh M, Ikeda Y, Nishio S, Nakadate T. Glycoprotein Ia/IIa-mediated activation-dependent platelet adhesion to collagen. *Thromb Res* 1992; 65: 165–176.
158 Moroi M, Onitsuka I, Imaizumi T, Jung SM. Involvement of ativated integrin $\alpha2\beta1$ in the firm adhesion of platelets onto a surface of immobilised collagen under flow conditions. *Thromb Haemost* 2000; 83: 769–776.
159 Kawaguchi S, Bergelson JM, Finberg RW, Hemler ME. Integrin alpha(2) cytoplasmic domain deletion effects – loss of adhesive activity parallels ligand-independent recruitment into focal adhesions. *Mol Biol Cell* 1994; 5: 977–988.
160 O'Toole TE, Ylanne J, Culley BM. Regulation of integrin affinity states through an NPXY motif in the beta-subunit cytoplasmic domain. *J Biol Chem* 1995; 270: 8553–8558.
161 Essex DW, Chen K, Swiatkowska M. Localization of protein disulfide isomerase to the external surface of the platelet plasma membrane. *Blood* 1995; 86: 2168–2173.
162 Chen K, Detwiler TC, Essex DW. Characterization of protein disulphide isomerase released from activated platelets. *Br J Haematol* 1995; 90: 425–431.
163 McLaughlin SH, Bulleid NJ. Thiol-independent interaction of protein disulphide isomerase with type X collagen during intra-cellular folding and assembly. *Biochem J* 1998; 331: 793–800.
164 Baksh S, Burns K, Andrin C, Michalak M. Interaction of calreticulin with protein disulfide isomerase. *J Biol Chem* 1995; 270: 31338–31344.
165 Burgess JK, Hotchkiss KA, Suter C et al. Physical proximity and functional association of Glycoprotein 1bα and protein-disulfide isomerase on the platelet plasma membrane. *J Biol Chem* 2000; 275: 9758–9766.
166 Hogg PJ, Hotchkiss KA, Jiminez BM, Stathakis P, Chesterman CN. Interaction of platelet-derived growth factor with thrombospondin 1. *Biochem J* 1997; 326: 709–716.
167 Essex DW, Li M. Protein disulphide isomerase mediates platelet aggregation and secretion. *Br J Haematol* 1999; 104: 448–454.
168 Essex DW, Li M. A polyclonal antibody to protein disulfide isomerase induces platelet aggregation and secretion. *Thromb Res* 1999; 96: 445–450.
169 O'Neill S, Robinson A, Deering A, Ryan M, Fitzgerald D, Moran N. The platelet integrin αIIb$\beta3$ has an endogenous thiol isomerase activity. *J Biol Chem* 2000; 275: 36984–36990.
170 Yan B, Smith JW. A redox site involved in integrin activation. *J Biol Chem* 2000; 275: 39964–39972.
171 Gofer-Dadosh N, Klepfish A, Schmilowitz H, Shaklai M, Lahav J. Affinity modulation in platelet $\alpha2\beta1$ following ligand binding. *Biochem Biophys Res Commun* 1997; 232: 724–727.
172 Lahav J, Gofer-Dadosh N, Luboshitz J, Hess O, Shaklai M. Protein disulfide isomerase mediates integrin-dependent adhesion. *FEBS Lett* 2000; 475: 89–92.
173 Chung J, Gao AG, Frazier WA. Thrombospondin acts via integrin-associated protein to activate the platelet integrin αIIb$\beta3$. *J Biol Chem* 1997; 272: 14740–14746.
174 Chung J, Wang XQ, Lindberg FP, Frazier WA. Thrombospondin-1 acts via IAP/CD47 to synergize with collagen in alpha 2 beta 1-mediated platelet activation. *Blood* 1999; 94: 642–648.
175 Frazier WA, Gao AG, Dimitry J et al. The thrombospondin receptor integrin-associated protein (CD47) functionally couples to heterotrimeric G(i). *J Biol Chem* 1999; 274: 8554–8560.
176 Yanaga F, Poole A, Asselin J et al. Syk interacts with tyrosine-phosphorylated proteins in human platelets activated by collagen and cross-linking of the Fcγ-IIA receptor. *Biochem J* 1995; 311: 471–478.
177 Gratacap M-P, Payrastre B, Viala C, Mauco G, Plantavid M,

Cambridge Books Online © Cambridge University Press, 2009

Chap H. Phosphatidylinositol 3,4,5-triphosphate-dependent stimulation of phospholipase C-γ2 is an early key event in FcγRIIA-mediated activation of human platelets. *J Biol Chem* 1998; 273: 24314–24321.

178 Sullam PM, Hyun WC, Szöllösi J, Dong J-F, Foss WM, López JA. Physical proximity and functional interplay of the Glycoprotein Ib–IX–V complex and the Fc receptor FcRIIA on the platelet plasma membrane. *J Biol Chem* 1998; 273: 5331–5336.

179 Poole A, Gibbins JM, Turner M et al. The Fc receptor γ-chain and the tyrosine kinase syk are essential for activation of mouse platelets by collagen. *EMBO J* 1997; 16: 2333–2341.

180 Gibbins J, Asselin J, Farndale RW, Barnes MJ, Law CL, Watson SP. Tyrosine phosphorylation of the Fc receptor γ-chain in collagen-stimulated platelets. *J Biol Chem* 1996; 271: 18095–18099.

181 Gibbins JM, Okuma M, Farndale RW, Barnes MJ, Watson SP. Glycoprotein VI is the collagen receptor in platelets which underlies tyrosine phosphorylation of the Fc receptor γ-chain. *FEBS Lett* 1997; 413: 255–259.

182 Tsuji M, Ezumi Y, Arai M, Takayama H. A novel association of Fc receptor γ-chain with glycoprotein VI and their co-expression as a collagen receptor in human platelets. *J Biol Chem* 1997; 272: 23528–23531.

183 Falati S, Edmead CE, Poole AW. Glycoprotein Ib–V–IX, a receptor for von Willebrand Factor, couples physically and functionally to the Fc receptor γ-chain, Fyn, and Lyn to activate human platelets. *Blood* 1999; 94: 1648–1656.

184 Zhu Q, Zelinka P, White T, Tanzer ML. Calreticulin-integrin bidirectional signalling complex. *Biochem Biophys Res Commun* 1997; 232: 354–358.

185 Coppolino MG, Woodside MJ, Demaurex N, Grinstein S, StArnaud R, Dedhar S. Calreticulin is essential for integrin-mediated calcium signalling and cell adhesion. *Nature* 1997; 386: 843–847.

186 Coppolino MG, Dedhar S. Ligand-specific, transient interaction between integrins and calreticulin during cell adhesion to extracellular matrix proteins is dependent upon phosphorylation dephosphorylation events. *Biochem J* 1999; 340: 41–50.

187 Kwon MS, Park CS, Choi K-R et al. Calreticulin couples calcium release and calcium influx in integrin-mediated calcium signalling. *Mol Biol Cell* 2000; 11: 1433–1443.

188 Coppolino MG, Leung-Hagesteijn C, Dedhar S, Wilkins J. Inducible interaction of integrin $\alpha 2\beta 1$ with calreticulin. *J Biol Chem* 1995; 270: 23132–23138.

189 Jackson DE, Kupcho KR, Newman PJ. Characterization of phosphotyrosine binding motifs in the cytoplasmic domain of platelet/endothelial cell adhesion molecule-1 (PECAM-1) that are required for the cellular association and activation of the protein-tyrosine phosphatase, SHP-2. *J Biol Chem* 1997; 272: 24868–24875.

190 Cicmil M, Thomas JM, Sage T et al. Collagen, convulxin and thrombin stimulate aggregation-independent tyrosine phosphorylation of CD31 in platelets. *J Biol Chem* 2000; 275: 27339–27347.

191 Patil S, Newman DK, Newman PJ. Platelet endothelial cell adhesion molecule-1 serves as an inhibitory receptor that modulates platelet responses to collagen. *Blood* 2001; 97: 1727–1732.

192 Zhao T, Newman PJ. Integrin activation by regulated dimerization and oligomerization of platelet endothelial cell adhesion molecule (PECAM)-1 from within the cell. *J Cell Biol* 2001; 152: 65–74.

193 Scherberich A, Moog S, Haan-Archipoff G, Azorsa DO, Lanza F, Beretz A. Tetraspanin CD9 is associated with very late-acting integrins in human vascular smooth muscle cells and modulates collagen matrix re-organisation. *Arterioscler Thromb Vasc Biol* 1998; 18: 1691–1697.

194 Indig FE, Diaz-Gonzalez F, Ginsberg MH. Analysis of the tetraspanin CD9-integrin alpha(IIb)beta(3; (GPIIb–IIIa; complex in platelet membranes and transfected cells. *Biochem J* 1997; 327: 291–298.

195 Longhurst CM, White MM, Wilkinson DA, Jennings LK. A CD9, alpha(IIb)beta 3, integrin-associated protein, and GPIb/V/IX complex on the surface of human platelets is influenced by alpha(IIb)beta 3 conformational states. *Eur J Biochem* 1999; 263: 104–111.

196 Miao W-M, Vasile E, Lane WS, Lawler J. CD36 associates with CD9 and integrins on human blood platelets. *Blood* 2001; 97: 1689–1696.

197 Ozaki Y, Satoh K, Kuroda K et al. Anti-CD9 monoclonal antibody activates $p72^{syk}$ in human platelets. *J Biol Chem* 1995; 270: 15119–15124.

198 Clemetson KJ, Polgar J, Clemetson JM. Snake venom C-type lectins as tools in platelet research. *Platelets* 1998; 9: 165–169.

199 Arocha-Pinango CL, Marchi R, Carvajal Z, Guerrero B. Invertebrate compounds acting on the hemostatic mechanism. *Blood Coagulation Fibrinol* 1999; 10: 43–68.

200 Andrews RK, Berndt MC. Snake venom modulators of platelet adhesion receptors and their ligands. *Toxicon* 2000; 38: 775–791.

201 Francischetti IMB, Ghazaleh FA, Reis RAM, Carlini CR, Guimaraes JA. Convulxin induces platelet activation by a tyrosine-kinase-dependent pathway and stimulates tyrosine phosphorylation of platelet proteins, including PLCγ2, independently of integrin $\alpha_{IIb}\beta_3$. *Arch Biochem Biophys* 1998; 353: 239–250.

202 De Luca M, Ward CM, Ohmori K, Andrews RK, Berndt MC. Jararhagin and jaracetin: novel snake venom inhibitors of the integrin collagen receptor, $\alpha_2\beta_1$. *Biochem Biophys Res Commun* 1995; 206: 570–576.

203 Kamiguti AS, Hay CRM, Zuzel M. Inhibition of collagen-induced platelet aggregation as the result of cleavage of $\alpha_2\beta_1$-integrin by the snake venom metalloproteinase jararhagin. *Biochem J* 1996; 320: 635–641.

204 Kamiguti AS, Markland FS, Zhou Q, Laing GD, Theakston RGD, Zuzel M. Proteolytic cleavage of the β_1 subunit of platelet $\alpha_2\beta_1$ integrin by the metalloproteinase Jararhagin compromises collagen-stimulated phosphorylation of pp72syk. *J Biol Chem* 1997; 272: 32599–32605.

205 Ivaska J, Käpylä J, Pentikäinen O et al. A peptide inhibiting the collagen binding function of integrin α_2I domain. *J Biol Chem* 1999; 274: 3513–3521.

Cambridge Books Online © Cambridge University Press, 2009

206 Pentikäinen O, Hoffrén A-M, Ivaska J et al. RKKH peptides from the snake venom metalloproteinase of *Bothrops jararaca* bind near the metal ion-dependent adhesion site of the human integrin $\alpha 2$ I-domain. *J Biol Chem* 1999; 274: 31493–31505.
207 Souza DHF, Iemma MRC, Ferreira LL et al. The disintegrin-like domain of the snake venom metalloprotease alternagin inhibits alpha 2 beta 1 integrin-mediated cell adhesion. *Arch Biochem Biophys* 2000; 384: 341–350.
208 Teng C-M, Ko F-N, Tsai I-H, Hung M-L, Huang T-F. Trimucytin: a collagen-like aggregating inducer isolated from *trimeresurus mucrosquamatus* snake venom. *Thromb Haemost* 1993; 69: 286–292.
209 Shin Y, Morita T. Rhodocytin, a functional novel platelet agonist belonging to the heterodimeric C-type lectin family, induces platelet aggregation independently of glycoprotein Ib. *Biochem Biophys Res Commun* 1998; 245: 741–745.
210 Chung C-H, Au L-C, Huang T-F. Molecular cloning and sequence analysis of aggretin, a collagen-like platelet aggregation inducer. *Biochem Biophys Res Commun* 1999; 263: 723–727.
211 Hers I, Berlanga O, Tiekstra MJ, Kamiguti AS, Theakston DG, Watson SP. Evidence against a direct role of the integrin $\alpha_2\beta_1$ in collagen-induced tyrosine phosphorylation in human platelets. *Eur J Biochem* 2000; 267: 2088–2097.
212 Inoue K, Ozaki Y, Satoh K et al. Signal transduction pathways mediated by glycoprotein Ia/IIa in human platelets: comparison with those of glycoprotein VI. *Biochem Biophys Res Commun* 1999; 256: 114–120.
213 Wang RH, Kini RM, Chung MCM. Rhodocetin, a novel platelet aggregation inhibitor from the venom of *Calloselasma rhodostoma* (Malayan pit viper): synergistic and noncovalent interaction between its subunits. *Biochemistry* 1999; 38: 7584–7593.
214 Eble JA, Beermann B, Hinz H-J, Schmidt-Hederich A. $\alpha 2\beta 1$ integrin is not recognised by rhodocytin, but is the specific, high-affinity target of rhodocetin, an RGD-independent disintegrin and potent inhibitor of cell adhesion to collagen. *J Biol Chem* 2001; 276: 12274–12284.
215 Zhou Q, Dangelmaier C, Smith JB. The hemorrhagin, catrocollastatin, inhibits collagen-induced platelet aggregation by binding to collagen via its disintegrin-like domain. *Biochem Biophys Res Commun* 1996; 219: 720–726.
216 Depraetere H, Kerekes A, Deckmyn H. The collagen-binding leech products rLAPP and calin prevent both von Willebrand factor and alpha2beta1(GPIa/IIa)-I-domain binding to collagen in a different manner. *Thromb Haemost* 1999; 82: 1160–1163.
217 Pignatelli P, Pulcinelli FM, Lenti L, Gazzaniga PP, Violi F. Vitamin E inhibits collagen-induced platelet activation by blunting hydrogen peroxide. *Arterioscler Thromb Vasc Biol* 1999; 19: 2542–7.
218 Ichinohe T, Takayama H, Ezumi Y et al. Collagen-stimulated activation of syk but not c-src is severely compromised in human platelets lacking membrane glycoprotein VI. *J Biol Chem* 1997; 272: 63–68.
219 Nakamura T, Jamieson GA, Okuma M, Kambayashi J-I, Tandon NN. Platelet adhesion to native type I collagen fibrils: role of GPVI in divalent cation-dependent and -independent adhesion and thromboxane A_2 generation. *J Biol Chem* 1998; 273: 4338–4344.
220 Nakamura T, Kambayashi J, Okuma M, Tandon NN. Activation of the GP IIb-IIIa complex induced by platelet adhesion to collagen is mediated by both $\alpha_2\beta_1$ integrin and GPVI. *J Biol Chem* 1999; 274: 11897–11903.
221 Blake RA, Schieven GL, Watson SP. Collagen stimulates tyrosine phosphorylation of phospholipase Cγ2 but not phospholipase Cγ1 in human platelets. *FEBS Lett* 1994; 353: 212–216.
222 Daniel JL, Dangelmaier C, Smith BJ. Evidence for a role for tyrosine phosphorylation of phospholipase Cγ2 in collagen induced platelet cytosolic calcium mobilization. *Biochem J* 1994; 302: 617–622.
223 Salzman EW, Levine L. Cyclic 3′,5′adenosine monophosphate in human blood platelets II. Effect of N^6-2′-O-dibutyryl cyclic 3′,5′-adenosine monophosphate on platelet function. *J Clin Invest* 1971; 50: 131–141.
224 Lapetina EG. Effect of pertussis toxin on the phosphodiesteric cleavage of the polyphosphoinositides by guanosine 5’-O-thiotriphosphate and thrombin in permeabilised human platelets. *Biochim Biophys Acta* 1986; 884: 219–224.
225 Farndale RW, Winkler AB, Martin BR, Barnes MJ. Inhibition of human platelet adenylate cyclase by collagen fibres. *Biochem J* 1992; 282: 25–32.
226 Wadman IA, Virdee K, Fernandez DS, Wasunna CL, Farndale RW. In Watson SP, Authi K eds. *Platelets: a practical approach.* Oxford: IRL press; 1996: 173–198.
227 Smith CCT, Stanyer L, Cooper MB, Betteridge DJ. Platelet aggregation may not be a prerequisite for collagen-stimulated platelet generation of nitric oxide. *Biochim Biophys Acta* 1999; 1473: 286–292.
228 Chiang TM, Wang YB, Kang ES. Role of the recombinant protein of the platelet receptor for type I collagen in the release of nitric oxide during platelet aggregation. *Thromb Res* 2000; 100: 427–432.
229 Ezumi Y, Shindoh K, Tsuji M, Takayama H. Physical and functional association of the Src family kinases Fyn and Lyn with the collagen receptor glycoprotein VI-Fc receptor γ chain complex on human platelets. *J Exp Med* 1998; 188: 267–276.
230 Briddon SJ, Watson SP. Evidence of the involvement of $p59^{fyn}$ and $p53/56^{lyn}$ in collagen receptor signalling in human platelets. *Biochem J* 1999; 338: 203–209.
231 Quek LS, Pasquet JM, Hers I et al. Fyn and lyn phosphorylate the Fc receptor γ chain downstream of glycoprotein VI in murine platelets, and lyn regulates a novel feedback pathway. *Blood* 2000; 96: 4246–4253.
232 Asazuma N, Yatomi Y, Ozaki Y et al. Protein-tyrosine phosphorylation and p72syk activation in human platelets stimulated with collagen is dependent upon glycoprotein Ia/IIa and actin polymerisation. *Thromb Haemost* 1996; 75: 648–654.
233 Asselin J, Gibbins JM, Achison M et al. A collagen-like peptide stimulates tyrosine phosphorylation of syk and phospholipase C γ2 in platelets independent of the integrin, $\alpha 2\beta 1$. *Blood* 1997; 89: 1235–1242.
234 Gross BS, Lee JR, Clements JL et al. Tyrosine phosphorylation

Cambridge Books Online © Cambridge University Press, 2009

of SLP-76 is downstream of Syk following stimulation of the collagen receptor in platelets. *J Biol Chem* 1999; 274: 5963–5971.
235 Asazuma N, Wilde JI, Berlanga O et al. Interaction of linker for activation of T cells with multiple adapter proteins in platelets activated by the glycoprotein VI-selective ligand, convulxin. *J Biol Chem* 2000; 275: 33427–33343.
236 Pasquet J-M, Quek L, Pasquet S et al. Evidence of a role for SHP-1 in platelet activation by the collagen receptor glycoprotein VI. *J Biol Chem* 2000; 275: 28526–28531.
237 Gibbins JM, Briddon S, Shutes A et al. The p85 subunit of phosphatidylinositol 3-kinase associates with the Fc receptor γ-chain and linker for activator of T cells (LAT) in platelets stimulated by collagen and convulxin. *J Biol Chem* 1998; 273: 34437–34443.
238 Banfic H, Downs CP, Rittenhouse SE. Biphasic activation of PKBα/Akt in platelets. Evidence for stimulation both by phosphatidylinositol 3,4-bisphosphate, produced via a novel pathway, and by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 1998; 273: 11630–11637.
239 Selheim F, Idsoe R, Fukami MH, Holmsen H, Vassbotn FS. Formation of PI 3-kinase products in platelets by thrombin, but not collagen, is dependent on synergistic autocrine stimulation, particularly through secreted ADP. *Biochem Biophys Res Commun* 1999; 263: 780–785.
240 Pasquet J-M, Bobe R, Gross B et al. A collagen-related peptide regulates phospholipase Cγ2 via phosphatidylinositol 3-kinase in human platelets. *Biochem J* 1999; 342: 171–177.
241 Falet H, Barkalow KL, Pivniouk VI, Barnes MJ, Geha RS, Hartwig JH. Roles of SLP-76, phosphoinositide 3-kinase and gelsolin in the platelet shape changes initiated by the collagen receptor GpVI/FcRγ-chain complex. *Blood* 2000; 96: 3786–3792.
242 Oda A, Ikeda Y, Ochs HD et al. Rapid tyrosine phosphorylation and activation of Bruton's tyrosine/Tec kinases in platelets induced by collagen binding or CD32 cross-linking. *Blood* 2000; 95: 1663–1670.
243 Quek LS, Bolen J, Watson SP. A role for Bruton's tyrosine kinase (Btk) in platelet activation by collagen. *Curr Biol* 1998; 8: 1137–1140.
244 Bauer M, Retzer M, Wilde JI et al. Dichotomous regulation of myosin phosphorylation and shape change by Rho-kinase and calcium in intact human platelets. *Blood* 1999; 94: 1665–1672.
245 Miranti CK, Leng L, Maschberger P, Brugge JS, Shattil SJ. Identification of a novel integrin signalling pathway involving the kinase Syk and the guanine nucleotide exchange factor Vav1. *Curr Biol* 1998; 8: 1289–1299.
246 Leo A, Schraven B. Networks in signal transduction: the role of adaptor proteins in platelet activation. *Platelets* 2000; 11: 429–445.
247 Heino J. The collagen receptor integrins have distinct ligand recognition and signalling functions. *Matrix Biol* 2000; 19: 319–323.
248 Koyama T, Raines EW, Bornfeldt KE, Roberts JM, Ross R. Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of cdk2 inhibitors. *Cell* 1996; 87: 1069–1078.
249 Keely PJ, Parise LV. The $\alpha2\beta1$ integrin is a necessary co-receptor for collagen-induced activation of syk and the subsequent phosphorylation of phospholipase Cγ2 in platelets. *J Biol Chem* 1996; 271: 26668–26676.
250 Haimovich B, Lipfert L, Brugge JS, Shattil SJ. Tyrosine phosphorylation and cytoskeletal reorganization in platelets are triggered by interaction of integrin receptors with their immobilized ligands. *J Biol Chem* 1993; 268: 15868–15877.
251 Polanowska-Grabowska R, Geanacopoulos M, Gear ARL. Platelet adhesion to collagen via the $\alpha_2\beta_1$ integrin under arterial flow conditions causes rapid tyrosine phosphorylation of pp125^{fak}. *Biochem J* 1993; 296: 543–547.
252 Achison M, Elton CM, Hargreaves PG et al. Integrin-independent tyrosine phosphorylation of p125fak in human platelets stimulated by collagen. *J Biol Chem* 2001; 276: 3167–3174.
253 Polanowska-Grabowska R, Simon CGJ, Falchetto R, Shabanowitz J, Hunt DF, Gear ARL. Platelet adhesion to collagen under flow causes dissociation of a phosphoprotein complex of heat-shock proteins and protein phosphatase 1. *Blood* 1997; 90: 1516–1526.
254 Mulrooney J, Foley K, Vineberg S, Barreuther M, Grabel L. Phosphorylation of the beta 1 integrin cytoplasmic domain: Toward an understanding of function and mechanism. *Exp Cell Res* 2000; 258: 332–341.
255 Heemskerk JWM, Vuist WMJ, Feijge MAH, Reutelingsperger CPM, Lindhout T. Collagen but not fibrinogen surfaces induce bleb formation, exposure of phosphatidylserine, and procoagulant activity of adherent platelets: Evidence for regulation by protein tyrosine kinase-dependent Ca^{2+} responses. *Blood* 1997; 90: 2615–2625.
256 Pasquet J-M, Dachary-Prigent J, Nurden AT. Microvesicle release is associated with extensive protein tyrosine dephosphorylation in platelet stimulated by A23187 or a mixture of thrombin and collagen. *Biochem J* 1998; 333: 591–599.
257 Heemskerk JWM, Siljander P, Bevers EM, Farndale RW, Lindhout T. Receptors and signalling mechanisms in the procoagulant response of platelets. *Platelets* 2000; 11: 301–306.
258 Heemskerk JWM, Siljander P, Vuist WMJ et al. Function of glycoprotein VI and integrin $\alpha2\beta1$ in the procoagulant response of single, collagen-adherent platelets. *Thromb Haemost* 1999; 81: 782–92.
259 Siljander P, Farndale RW, Feijge MAH et al. Platelet adhesion enhances the glycoprotein VI-mediated procoagulant response: involvement of p38 MAP kinase and calpain. *Arterioscler Thromb Vasc Biol* 2001; 21: 618.
260 Saklatvala J, Rawlinson L, Waller RJ et al. Role for p38 mitogen-activated protein kinase in platelet activation caused by collagen or a thromboxane analogue. *J Biol Chem* 1996; 271: 6586–6589.
261 Ivaska J, Reunanen H, Westermarck J, Koivisto L, Kähäri V-M, Heino J. Integrin $\alpha_2\beta_1$ mediates isoform-specific activation of p38 and upregulation of collagen gene transcription by a mechanism involving the α_2 cytoplasmic tail. *J Cell Biol* 1999; 147: 401–415.
262 Kamiguti AS, Theakston RDG, Watson SP, Bon C, Laing GD, Zuzel M. Distinct contributions of glycoprotein VI and alpha(2)beta(1) integrin to the induction of platelet protein

Cambridge Books Online © Cambridge University Press, 2009

tyrosine phosphorylation and aggregation. *Arch Biochem Biophys* 2000; 374: 356–362.
263 Piguet PF, Vesin C, Donati Y, Tacchini-Cottier F, Belin D, Barazzone C. Urokinase receptor (uPAR, CD87; is a platelet receptor important for kinetics and TNF-induced endothelial adhesion in mice. *Circulation* 1999; 99: 3315–3321.
264 Dorahy DJ, Lincz LF, Meldrum CJ, Burns GF. Biochemical isolation of a membrane microdomain from resting platelets highly enriched in the plasma membrane glycoprotein CD36. *Biochem J* 1996; 319: 67–72.
265 Dorahy DJ, Burns GF. Active Lyn protein tyrosine kinase is selectively enriched within membrane microdomains of resting platelets. *Biochem J* 1998; 333: 373–379.
266 Valentino LA, Ladisch S. Tumor gangliosides enhance $\alpha_2\beta_1$ integrin-dependent platelet activation. *Biochim Biophys Acta* 1996; 1316: 19–28.
267 Wen F-Q, Jabbar AA, Patel DA, Kazarian T, Valentino LA. Atherosclerotic aortic gangliosides enhance integrin-mediated platelet adhesion to collagen. *Arterioscler Thromb Vasc Biol* 1999; 19: 519–524.
268 Beer JH, Rabaglio M, Berchtold P et al. Autoantibodies against the platelet glycoproteins (GP) IIb/IIIa, Ia/IIa and IV and partial deficiency in GPIV in a patient with a bleeding disorder and a defective platelet collagen interaction. *Blood* 1993; 82: 820–829.
269 Ruggeri ZM, Dent JA, Saldívar E. Contribution of distinct adhesive interactions to platelet aggregation in flowing blood. *Blood* 1999; 94: 172–178.
270 Wu Y-P, Vink T, Schiphorst N et al. Platelet thrombus formation on collagen at high shear rates is mediated by von Willebrand factor-glycoprotein Ib interaction and inhibited by von Willebrand factor-glycoprotein IIb/IIIa interaction. *Arterioscler Thromb Vasc Biol* 2000; 20: 1661–1667.
271 Kulkarni S, Dopheide SM, Yap CL et al. A revised model of platelet aggregation. *J Clin Invest* 2000; 105: 783–791.
272 Savage B, Ginsberg MH, Ruggeri ZM. Influence of fibrillar collagen structure on the mechanisms of platelet thrombus formation under flow. *Blood* 1999; 94: 2704–2715.
273 Monnet E, Sizaret PY, Arbeille B, Fauvel-Lafève F. Different role of platelet glycoprotein Ia/IIa in contact and activation induced by type I and type III collagens. *Thromb Res* 2000; 98: 423–433.
*1 Andrews RK, Shen Y, Gardiner EE, Berndt MC. Platelet adhesion receptors and (patho)physiological thrombus formation. *Histol Histopathol* 2001; 16: 969–90.
*2 Clemetson KJ, Clemetson JM. Platelet collagen receptors. *Thromb Haemost* 2001; 86: 189–197.
*3 Fitzgerald J, Bateman JF. A new FACIT of the collagen family: COL21A1(1). *FEBS Lett* 2001; 505: 275–280.
*4 Reiner AP, Siscovick DS, Rosendaal FR. Platelet glycoprotein gene polymorphisms and risk of thrombosis: facts and fancies. *Rev Clin Exp Hematol* 2001; 5: 262–287.
*5 Santoso S. Platelet polymorphisms in thrombotic disorders. *Transfus Clin Biol* 2001; 8: 261–266.
*6 Kunicki TJ. The role of platelet collagen receptor (glycoprotein Ia/IIa; integrin alpha2 beta1) polymorphisms in thrombotic disease. *Curr Opin Hematol* 2001; 8: 277–285.
*7 Kunicki TJ, Ruggeri ZM. Platelet collagen receptors and risk prediction in stroke and coronary artery disease. *Circulation* 2001; 104: 1451–1453.
*8 Moog S, Mangin P, Lenain N et al. Platelet glycoprotein V binds to collagen and participates in platelet adhesion and aggregation. *Blood* 2001; 98: 1038–1046.
*9 Nieswandt B, Brakebusch C, Bergmeier W et al. Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. *EMBO J* 2001; 20: 2120–2130.
*10 Xiong J-P, Stehle T, Diefenbach B et al. Crystal structure of the extracellular segment of integrin $\alpha V\beta 3$. *Science* 2001; 294: 339–345.
*11 Lagrue-Lak-Hal AH, Debili N, Kingbury G et al. Expression and function of the collagen receptor GPVI during megakaryocyte maturation. *J Biol Chem* 2001; 276: 15316–15325.
*12 Chen H, Locke D, Liu Y, Liu C, Kahn ML. The platelet receptor GPVI mediates both adhesion and signaling responses to collagen in a receptor density-dependent fashion. *J Biol Chem* 2001; 26: 26.
*13 Furihata K, Clemetson KJ, Deguchi H. Kunicki TJ. Variation in human platelet Glycoprotein VI content modulates Glycoprotein VI-specific prothrombinase activity. *Arterioscler Thromb Vasc Biol* 2001; 21: 1857–1863.
*14 Monnet E, Depraetere H, Legrand C, Deckmyn H, Fauvel-Lafeve F. A monoclonal antibody to platelet type III collagen-binding protein (TIIICBP) binds to blood and vascular cells, and inhibits platelet vessel-wall interactions. *Thromb Haemost* 2001; 86: 694–701.
*15 Jung SM, Moroi M. Activation of the platelet collagen receptor integrin alpha(2)beta(1): its mechanism and participation in the physiological functions of platelets. *Trends Cardiovasc Med* 2000; 10: 285–292.
*16 Essex DW, Li M, Miller A, Feinman RD. Protein disulfide isomerase and sulfhydryl-dependent pathways in platelet activation. *Biochemistry* 2001; 40: 6070–6075.
*17 Henshall TL, Jones KL, Wilkinson R, Jackson DE. Src homology 2 domain-containing protein-tyrosine phosphatases, SHP-1 and SHP-2, are required for platelet endothelial cell adhesion molecule-1/CD31-mediated inhibitory signaling. *J Immunol* 2001; 166: 3098–3106.
*18 Ohmori T, Yatomi Y, Wu Y et al. Wheat germ agglutinin-induced platelet activation via Platelet Endothelial Cell Adhesion Molecule-1: involvement of rapid phospholipase Cγ2 activation by src family kinases. *Biochemistry* 2001; 40: 12992–13001.
*19 Watson SP, Asazuma N, Atkinson B et al. The role of ITAM- and ITIM-coupled receptors in platelet activation by collagen. *Thromb Haemost* 2001; 86: 276–288.
*20 Jones KL, Hughan SC, Dopheide SM et al. Platelet Endothelial Cell Adhesion Molecule-1 is a negative regulator of platelet-collagen interactions. *Blood* 2001; 98: 1456–1463.
*21 Mahooti S, Graesser D, Patil S et al. PECAM-1 (CD31) expression modulates bleeding time in vivo. *Am J Pathol* 2000; 157: 75–81.
*22 Judd BA, Koretzky GA. The role of the adapter molecule SLP-76 in platelet function. *Oncogene* 2001; 20: 6291–6299.

Cambridge Books Online © Cambridge University Press, 2009

12

Platelet receptors: von Willebrand factor

Jerry Ware and Zaverio M. Ruggeri

Division of Experimental Hemostasis and Thrombosis
Departments of Molecular and Experimental Medicine and of Vascular Biology
The Scripps Research Institute, La Jolla, CA, USA

Historical perspective and overview

For most bleeding disorders, the symptoms and clinical recognition of the disease occurred long before the biochemical characterization of the disease. A case in point is the description of von Willebrand disease by the Finnish physician Erik von Willebrand (ca. 1926) and the description of the Bernard–Soulier syndrome by French physicians, Jean Bernard and Jean-Pierre Soulier (ca. 1948). In both cases, decades would past before the molecular basis of the disease would define roles for von Willebrand factor (vWF) and the platelet receptor, glycoprotein (GP) Ib–IX–V, in hemostasis and thrombosis. Two-dimensional gel electrophoresis would associate an absent platelet glycoprotein in patients with the Bernard–Soulier syndrome[1]; while the molecular identity of vWF would not be unequivocally established until the gene was characterized and shown to be a distinct protein circulating in a complex with blood coagulation factor VIII[2–4].

The current models of platelet adhesion to the extracellular matrix involve a number of different ligands and platelet receptors coordinated in a synergistic response to correct a vascular lesion. Nevertheless, vWF and its platelet receptors represent a major component in this response and become the exclusive ligand and receptor interaction under blood flow conditions typically found in arterioles and small arteries. Thus, under certain conditions vWF-dependent thrombus formation is part of the synergistic response, yet under specific rheological conditions it becomes the sole ligand capable of initiating platelet deposition and thrombus growth.

The molecular mechanisms by which vWF participates in hemostasis can be viewed as an initial tethering of platelets to a reactive surface via the platelet GP Ib–IX–V receptor (Fig. 12.1). Through this transient and reversible interaction with vWF, platelets can become activated increasing the binding competency of the platelet integrin receptor GP IIb–IIIa ($\alpha_{IIb}\beta_3$). The combination of vWF interacting with both GP Ib–IX–V and $\alpha_{IIb}\beta_3$ results in stable and irreversible platelet attachment to the surface. Subsequently, the process can be repeated with the newly formed layer of platelets behaving as a reactive surface recruiting additional platelets and leading to thrombus growth. In this regard, the initial platelet adhesion and subsequent aggregation can both be viewed from a similar mechanistic standpoint, that is, both require platelet attachment to a reactive surface.

Although GP Ib–IX–V represents the primary adhesive platelet receptor for vWF, the role of $\alpha_{IIb}\beta_3$ in the sequence of events leading to thrombus formation should not be forgotten. A detailed description of $\alpha_{IIb}\beta_3$ can be found in Chapter 12, as it is the sole platelet receptor for fibrinogen. This chapter will focus on the structure and biological properties of the GP Ib–IX–V complex but will reference $\alpha_{IIb}\beta_3$ as it pertains to vWF-dependent thrombus formation (p. 183).

Structure

Table 12.1 summarizes the alternative nomenclature used for the subunits and genes encoding the GP Ib–IX–V complex. Most commonly, the subunits are referred to as GP Ib, GP IX and GP V (Fig. 12.1). The GP Ib molecule is actually composed of two disulfide-linked subunits, α and β (GP Ibα and GP Ibβ), both the products of separate genes. GP Ibα is the structurally and functionally dominant subunit of the complex (M_r, 145 000) and associates with

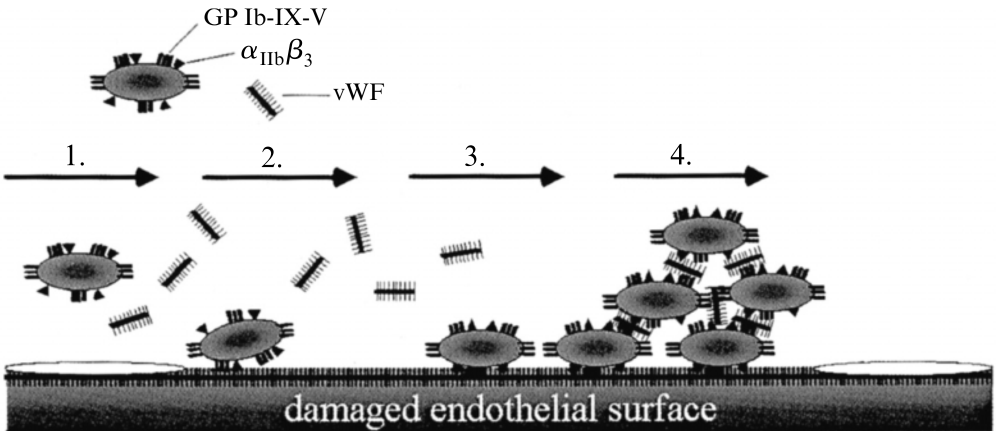


Fig. 12.1. Temporal events in vWF-dependent thrombus formation. In a regulated series of events, platelets will adhere to damaged endothelium and form a platelet thrombus. Depicted are a series of steps (1 to 4) representing a simplistic interpretation of the processes necessary to capture a platelet from flowing blood and build the platelet thrombus. (Step 1) A circulating platelet is traveling (left to right) and contains surface-expressed GP Ib–IX–V and GP IIb–IIIa ($\alpha_{IIb}\beta_3$) receptors. (Step 2) Via a transient and reversible interaction between platelet GP Ib–IX–V and von Willebrand factor (vWF), platelets will tether and move along the thrombogenic surface until the platelet undergoes activation. (Step 3) Upon activation the ligand binding competency of the $\alpha_{IIb}\beta_3$ receptor is increased leading to irreversible platelet adhesion. (Step 4) A subsequent repeat of steps 1–3 leads to the additional recruitment of platelets with the net effect of a growing thrombus on the thrombogenic surface. This figure illustrates the exclusive role of vWF and its receptors under flow conditions typically found in vessels with high shear rate, such as small arteries and arterioles. Under conditions when the blood flow rate is slower, vWF-dependent thrombus formation becomes part of a synergistic effort involving additional platelet receptors and ligands.

Table 12.1. Platelet von Willebrand factor receptors

GP receptor complex	Subunit composition	CD nomenclature	Genbank acc. number	HUGO[a] nomenclature	Chromosome location
GP Ib–IX–V	GP Ibα	CD42b	M22403	GP1BA	17pter–p12
	GP Ibβ	CD42c	Z23091	GP1BB	22q11.21–q11.23
	GP IX	CD42a	U07983	GP9	3q21
	GP V	CD42d	M80478	GP5	3q29
GP IIb–IIIa	GP IIb (αIIb)	CD41b	J02764,M34480	ITGA2B	17q21.32
	GP IIIa (β3)	CD61	J02703	ITGB3	17q21.32

Note:
[1] Human gene nomenclature http://www.gene.ucl.ac.uk/nomenclature]

GP Ibβ (M_r, 22000) and GP IX (M_r, 17000) in a 1: 1: 1 stoichiometry. GP V (M_r, 82000) associates with the GP Ib–IX complex in a 1:2 ratio (GP V: GP Ib–IX). The primary sequence of each of the four subunits has been quite revealing for identification of distinct domains, yet most characterizations of the complex have focused on GP Ibα since it contains binding sites for both vWF and α-thrombin (Fig. 12.2).

An amino-terminal 45 kDa domain of GP Ibα can be generated through limited tryptic digestion and retains vWF and α-thrombin binding properties[5–7]. A striking feature of the GP Ibα 45-kDa domain is the presence of seven leucine-rich repeats, which are found in a large number of seemingly unrelated proteins[8]. A single repeat is composed of 24 residues with leucines commonly found at positions 2, 5, 7, 12, 16, 21 and 24. Members of the leucine-rich repeat family have roles in signal transduction either as membrane receptors or as cytoplasmic signalling molecules[9]. The presence of a single leucine repeat in GP IX and GP Ibβ along with multiple repeats in GP V suggests the leucine-rich repeats may be critical for assembly and surface expression of the intact complex[10], a concept supported by mutations within the repeats of GP Ibα and GP IX which result in the Bernard–Soulier phenotype[11].

Cambridge Books Online © Cambridge University Press, 2009

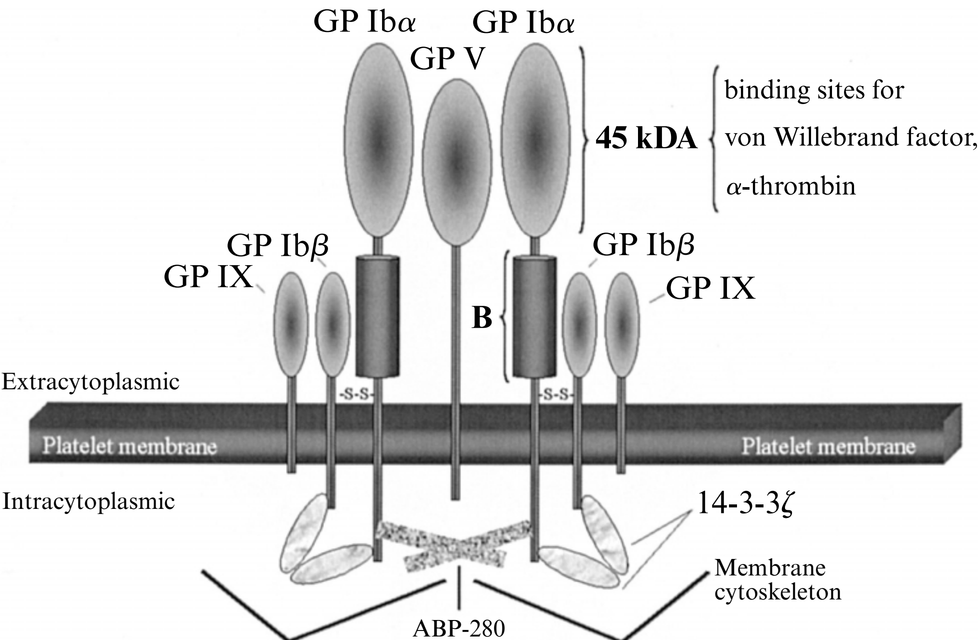


Fig. 12.2. The platelet GP Ib–IX–V complex. The GP Ib–IX–V complex exists on the platelet surface with a suggested stoichiometry of 2:2:2:1 (GP Ibα:GP Ibβ:GP IX:GPV). The α-subunit of GP Ib (GP Ibα) contains the ligand binding sites for vWF and α-thrombin within an amino-terminal 45 kDa domain. In addition, GP Ibα contains a large macroglycopeptide domain (*b*) that supports surface exposure of the α-subunit. Cytoplasmic interactions with the complex include ABP-280 (filamin) within the platelet membrane cytoskeleton and the 14-3-3ζ isoform of phospholipase A2. The three-dimensional structure of the complex or isolated domains of the complex have not been determined but the illustrated subunit sizes do correspond with their molecular weight contribution to the complex. The depicted diagram should be considered a conceptual cartoon of the GP Ib–IX–V complex.

The 45 kDa domain of GP Ibα can be functionally recapitulated via domain-specific expression of a secreted soluble receptor fragment[7,12]. This work has led to the identification of a series of negatively charged amino acids within the linear sequence of GP Ibα that are critical for vWF binding. Three tyrosine residues whose modification via a post-translational sulfation event also contribute to the negative charge of the linear sequence and are critical for binding to vWF[13,14].

The extracellular sequences of GP IX and GP Ibβ are similar to each other and are considerably smaller than GP Ibα. The lack of ligand binding sites within the extracellular sequences of either subunit seems to suggest their primary role in the complex is to support efficient intracellular assembly of the complex and translocation to the plasma membrane[15]. Each subunit of the GP Ib–IX–V complex has a single transmembrane domain. Recently, the cytoplasmic tails of GP Ibα and GP Ibβ have become areas of active research with the identification of a binding site for platelet phospholipase A2 (ζ-isoform of the 14–3–3 protein)[16,17]; filamin (actin-binding protein 280) binds the cytoplasmic tail of GP Ibα[18] and the biologic relevance of these interactions will be discussed below (p. 184).

A number of polymorphisms have been identified within the subunits of the GP Ib–IX–V complex. Most of the polymorphisms are associated with GP Ibα reflecting the more intense analysis and larger size of the GP Ibα gene. Most striking are the molecular weight variants visible by PAGE analysis which result from multiple replications of a 13-residue motif within the heavily glycosylated extracellular domain[19,20] (Fig. 12.2). A polymorphic amino acid, residue 145, within the 45 kDa domain of GP Ibα forms the basis for an alloantigen associated with platelet transfusion refractoriness[21,22].

Gene structure and expression

All four genes of the GP Ib–IX–V complex have a relatively simple organization with the majority of protein-encoding sequences present within a single exon. Detailed analysis for the promoters of GP Ibα and GP IX have been

Cambridge Books Online © Cambridge University Press, 2009

performed and have identified essential GATA-1 and Ets cis-acting elements within the core promoter of each gene[23,24]. These results have been confirmed in GATA-1 deficient mice which have a sevenfold reduction in GP Ibα gene expression and have supported the hypothesis that GATA and Ets binding sites are major regulatory elements for megakaryocytic-specific gene expression[25,26]. Also relevant to the expression of GP Ibα is a polymorphic Kozak sequence which can alter the levels of expressed GP Ib–IX–V complex. Circulating platelets with genetically predetermined levels of GP Ib–IX–V may influence an individual's susceptibility for the development of cardiovascular disease although population studies will be necessary to convincingly establish a correlation[27–29].

The expressed gene products of GP Ibα, GP Ibβ and GP IX assemble within the endoplasmic reticulum prior to being transported to the Golgi and the cell surface[30]. The role of GP Ibβ and GP IX in this process is to direct GP Ibα away from lysosomal degradation pathway and protect it from proteolysis[30]. This observation provides a mechanistic basis for how mutations within either the GP Ibβ or GP IX genes can abrogate the functional surface expression of the GP Ibα subunit. Of note, GP V is not a required component for assembly and expression of a GP Ib–IX complex[15], but may regulate the ability of a GP Ib–IX or GP Ib–IX–V complex to bind vWF[31,32]. The absence of mouse GP V within the murine GP Ib–IX–V complex does not result in a murine Bernard–Soulier syndrome, nor have mutations within the human GP V gene been associated with the Bernard–Soulier phenotype[33,34]. Thus, the physiologic relevance of GP V expression is not obvious and remains to be established. However, as a known thrombin substrate, GP V may contribute in the platelet response to thrombin[33,35].

A source of great confusion is the possibility of GP Ib–IX–V gene expression in cells of nonhematopoietic lineage. In particular, several reports from the same laboratory have identified an expressed GP Ib–IX complex on cultured endothelial cells[36–39], but these studies have not proposed an in vivo consequence of expression at these nonhematopoietic sites. However, the in vitro expression of GP Ib–IX by cultured endothelial cells is not without controversy, as others have reported an inability to identify a GP Ib–IX complex on cultured endothelial cells[40]. Moreover, in vivo studies of murine endothelial cells have failed to identify an endothelial GP Ib–IX–V complex[41]. What is clear is vWF can bind to endothelial cells via the integrin receptor, $\alpha_v\beta_3$ (40), or by electrostatic charges presented by glycosaminoglycans on the endothelial cell surface[42].

Congenital deficiencies

The Bernard–Soulier syndrome

Although rare, this congenital disease may be severe with life-threatening bleeding episodes occurring from early childhood[11]. The bleeding is caused by an absent or dysfunctional GP Ib–IX–V complex and is clinically characterized by an inability of platelets to agglutinate in the presence of ristocetin and macrothrombocytopenia. The Bernard–Soulier syndrome is inherited as an autosomal recessive trait and is associated with a growing database of heterogeneous mutations within GP Ibα, GP Ibβ and GP IX [http://www.bernard–soulier.org/]. Overall, the published studies have supported a concept that the syndrome parallels congenital abnormalities in other multisubunit receptor complexes, to the extent that a single subunit prevents assembly and surface expression of the entire complex. A mouse model of the Bernard–Soulier syndrome recapitulates all of the known characteristics of the human syndrome[43]. There is a limited number of missense mutations which do not abrogate the surface expression of the complex but still result in a dysfunctional GP Ib–IX–V receptor. How these variant complexes generate macrothrombocytopenia is still unclear, but is assumed to be related to the expression of a structurally abnormal GP Ib–IX–V complex.

Platelet-type von Willebrand disease

Nature has provided two bleeding disorders resulting in an altered affinity between vWF and platelet GP Ib–IX–V. The disease states, platelet type von Willebrand disease and type 2B von Willebrand disease, are abnormalities within the receptor or the ligand, respectively. Both result from missense mutations resulting in variant proteins capable of supporting an in vivo interaction between plasma vWF and platelets. In both cases, there is sufficient affinity to promote intravascular clumping, thrombocytopenia and a resulting bleeding disorder. These disorders have been model examples of mechanisms that regulate the interaction of vWF with GP Ib–IX–V. To date, two different mutations have been described that result in platelet-type von Willebrand disease and both reside within a limited region of GP Ibα[44]. It remains to be established which amino acids of vWF or GP Ibα are in direct contact with each other, but current hypotheses suggest that the phenotype in type 2B von Willebrand disease is due to amino acid substitutions which expose an otherwise cryptic binding site within the domain of vWF binding to GP Ib–IX–V[45]. A similar model for platelet-type von Willebrand disease would be that

Cambridge Books Online © Cambridge University Press, 2009

alterations in the receptor are able to interact with a normally cryptic binding site within the ligand. Both platelet-type von Willebrand disease and type 2B von Willebrand disease are two excellent experimental models to understand the physiologic events that regulate the interaction of platelets with vWF.

Biologic function

Ligand binding – vWF

To mediate platelet adhesion and aggregation during thrombus formation platelets must form stable bonds with components of the extracellular matrix (adhesion) or with other platelets (aggregation). For both adhesion and aggregation to occur, the platelet must adhere to a reactive surface and resist the force of flowing blood that would move the platelet further through the bloodstream[46]. To achieve this biological function platelets have two receptors recognizing vWF, the GP Ib–IX–V complex and the integrin $\alpha_{IIb}\beta_3$[47,48]. In the case of vWF binding to platelet receptors a model has emerged in which the GP Ib–IX–V complex and $\alpha_{IIb}\beta_3$ synergistically function in a temporal sequence of events[49]. A transient tethering of platelets to a thrombogenic surface requires the GP Ib–IX–V complex and results in reversible adhesion[50]. During this transient interaction the $\alpha_{IIb}\beta_3$ receptor may become activated resulting in the stable arrest of the platelet from flowing blood[46,51]. The same process can be repeated to capture flowing platelets onto the surface created by the already adherent platelets. The net result is a thrombus formed by platelet aggregates. Fibrinogen contributes to the stabilization of the forming thrombus, even under conditions where vWF was responsible for the initiation of platelet recruitment[46,52].

A number of experimental models have been developed to analyse the interaction of vWF with platelets. Historically, the use of the antibiotic ristocetin or the snake venom protein, botrocetin[53], have been widely used with stirred platelets in the aggregometer. In the stirred aggregometer, soluble vWF supports platelet–platelet interactions mediated exclusively through GP Ib–IX–V and not dependent upon the $\alpha_{IIb}\beta_3$ receptor. Alternatively, an agonist independent method of platelet aggregation can be demonstrated with soluble vWF and platelets in conditions generating high shear stress, but this event occurs only during high-shear and requires platelet activation and engagement of $\alpha_{IIb}\beta_3$[54]. In contrast, recently developed in vitro models of thrombus formation in flowing blood have documented surface-bound vWF as the exclusive ligand when flow conditions mimic those commonly found in arterioles or small arteries. The vWF-dependent mechanisms that support platelet tethering and irreversible platelet attachment in the presence of flow, can also function in the absence of flow[48]. However, in vivo this latter process is part of a synergistic response involving a number of different collagen platelet receptors and $\alpha_{IIb}\beta_3$ binding to fibrinogen. Thus, under these parameters the contribution of vWF is less obvious. Relative to future work, it is imperative that conclusions be referenced to the specific experimental assay, as the choice of modulator, chemical or mechanical, influences the relevance of the conclusion to in vivo platelet function.

Ligand binding – thrombin

Thrombin binding to GP Ib is well documented although the relevance of this binding and its contribution to the thrombin activation of platelets is more controversial. The identification of GP Ib as a thrombin binding site on the platelet surface predates the characterization of the platelet protease-activated receptors (PAR-1 and PAR-4)[55,56]. Originally characterized as the high-affinity platelet binding receptor for thrombin[57], later studies have shed doubt as to whether GP Ib should be considered the high-affinity thrombin binding site on platelets[58] and whether other factors, such as temperature, platelet–platelet interactions, internalization of the receptor or the inclusion of GP V into the complex can contribute to the formation of a high affinity binding site[33,59]. Regardless of these controversies, the 45 kDa amino-terminal domain of GP Ibα does contain a discrete linear amino acid sequence with homologies to other thrombin binding proteins, such as hirudin and the PARs, functioning as a distinct thrombin binding site[14,60,61]. A number of different reagents recognizing this linear sequence, such as monoclonal antibodies and the snake lectin echicetin, block thrombin binding to GP Ib and have provided the definitive proof of the receptor's thrombin binding properties[6].

A fundamental difference in thrombin binding to GP Ib vs. binding to the platelet PARs, is that GP Ib is not cleaved by the bound thrombin, whereas thrombin binding to PARs is associated with proteolytic cleavage and platelet activation (see Chapter 8). The PARs are important for thrombin-induced platelet activation, but a role for GP Ib is suggested in the initial stages of hemostasis when sub-optimal levels of thrombin are present[61]. Thrombin binding to GP Ib may also generate a procoagulant environment on the platelet surface as suggested by studies documenting the ability of monoclonal antibodies which block thrombin binding to GP Ib, to also inhibit annexin V

Cambridge Books Online © Cambridge University Press, 2009

binding to the platelet, one characteristic of a procoagulant membrane[62]. Thus, thrombin binding to GP Ib may generate an outer platelet membrane surface with procoagulant activity where prothrombin is cleaved by factor Xa to thrombin. Again, the molecular mechanisms are unresolved, but these observations are intriguing and may provide some hint for the physiologic relevance of thrombin binding to the GP Ib–IX–V complex.

Cytoplasmic interactions

The cytoplasmic domains on the α- and β-subunits of GP Ib associate with a number of intracytoplasmic molecules and presumably contribute to some undefined signalling pathways leading to integrin activation. One candidate element is a member of the 14–3–3 family of protein, specifically the ζ-isoform, 14–3–3ζ[63]. A binding site for 14–3–3ζ is located in the carboxyl-terminus of GP Ibα and the binding is regulated by phosphorylation of GP Ibα at serine[609 64]. The expression of a GP Ib–IX lacking the 14–3–3ζ binding site within GP Ibα reduces $\alpha_{IIb}\beta_3$ activation, a result confirmed by a 14–3–3ζ dominant-negative mutant's ability to inhibit vWF-induced integrin activation[65,66]. GP Ibβ also contributes to the binding of 14–3–3ζ and this binding is regulated by protein kinase A[17,67]. Binding sites on both GP Ibα and GP Ibβ may reflect the fact that dimeric 14–3–3ζ interacts with both subunits of GP Ib[67]. A model for the importance of 14–3–3ζ binding to GP Ib is based on the release of 14–3–3ζ from GP Ib which coincides with the ability of platelets to aggregate in the shear aggregometer. Moreover, blocking 14–3–3ζ release from the tail of GP Ib is associated with the inhibition of aggregation[67]. Thus, signalling through the cytoplasmic tail of GP Ib seems plausible given the candidate interactions with known signalling molecules, but the downstream effectors that ultimately affect integrin activation have not been identified.

A second critical interaction site within the GP Ibα cytoplasmic tail is a region binding to filamin (also called actin binding protein or ABP-280). This interaction cross-links GP Ib–IX–V to actin filaments within the plasma membrane cytoskeleton. The interaction of GP Ib–IX–V with the membrane skeleton may have an important role in regulating vWF/GP Ib–IX–V interaction. Recent studies have demonstrated that pretreating platelets with cytochalasin D enhances shear-induced platelet aggregation and reduces the threshold required for platelet aggregation[68,69]. Moreover, Chinese hamster ovary cells with surface-expressed mutant complexes have also demonstrated the linkage between the GP Ibα and filamin is critical for the cytochalasin effect on GP Ib–IX–V mediated aggregation[68].

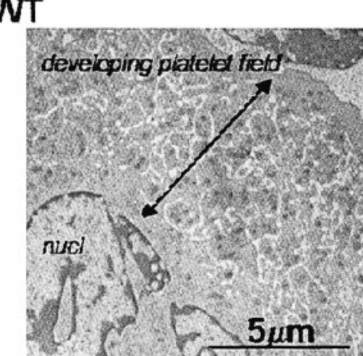
Platelets and inflammation of the endothelium

The platelet–subendothelial interaction via vWF is well established. However, recent studies have demonstrated a role for platelet GP Ibα in platelet–endothelium interaction in veins[42]. In normal conditions platelets do not interact with the endothelium. However, during inflammation platelets can adhere to the activated endothelial surface. Studies have suggested GP Ibα may be a counterreceptor for P-selectin and potentially mediate platelet rolling on the stimulated endothelium[70]. In vivo studies have documented rolling platelets on activated endothelium via a process that is dependent upon platelet GP Ibα and vWF[42]. The physiological relevance of this interaction is that it could cause platelet activation, which in turn generates a wide variety of platelet responses and potentially leads to venous thrombosis. Additional studies may help clarify the physiologic impact of platelet GP Ibα interactions with the intact endothelial surface.

Megakaryocytopoiesis

Beyond its direct participation in platelet adhesive functions, the GP Ib–IX–V complex also participates in megakaryocytopoiesis and platelet morphogenesis. The congenital absence of GP Ib–IX–V produces a 'giant' platelet phenotype. A mouse model of the Bernard–Soulier syndrome has provided proof of principle with a direct link between the absent receptor and impaired megakaryocytopoiesis[43]. The results demonstrate that in the absence of a GP Ib–IX–V complex the mature megakaryocyte fails to develop a normal demarcation membrane system or display developing platelet fields in the cytoplasm (Fig. 12.3). A disordered membrane system in the absence of GP Ib–IX–V alters the normal platelet release from the megakaryocyte and results in a mild thrombocytopenia with 'giant' platelets, or macrothrombocytopenia. The molecular basis for the abnormal megakaryocytopoiesis may be linked to the cytoplasmic interactions between the GP Ibα subunit and components of the platelet membrane cytoskeleton, but may also be influenced by extracytoplasmic components of the complex as antibodies to GP Ib–IX can alter proplatelet formation in vitro[71]. Moreover, there is in vitro evidence that the expression of GP Ib–IX–V in heterologous cells can affect cell proliferation and by analogy might also regulate megakaryocyte growth[72]. Future studies will have to define the molecular basis of the aberrant megakaryocyte morphology and the subsequent impairment of normal platelet release from the mature megakaryocyte.

Cambridge Books Online © Cambridge University Press, 2009



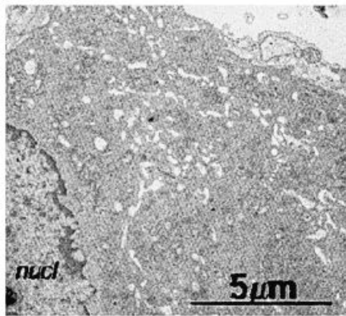


Fig. 12.3. Transmission electron microscopy of megakaryocytes devoid of platelet GP Ibα. Bone marrow from mouse femurs was isolated and prepped for transmission electron microscropy. Shown is a portion of mature megakaryocyte cytoplasm from a normal mouse (WT) and a GP Ibα-deficient mouse (GP Ibα^{null}). Nuclear material is shown (*nucl*) along with a developing platelet field and demarcation membrane system in the normal megakaryocyte cytoplasm. GP Ibα-deficient animals have mature megakaryocytes devoid of a normal developing platelet field and display a vacuolated or disordered demarcation membrane system ultimately leading to the characteristic macrothrombocytopenia of the Bernard–Soulier syndrome[43]. (Copyright 2000 National Academy of Sciences, USA.)

Conclusions

Several broad areas of work on the GP Ib–IX–V complex have the potential to provide some exciting results in the new millennium. First, will be a dissection of the molecular mechanisms whereby the complex participates in platelet activation. We have outlined studies highlighting the potential regulatory mechanisms that control vWF binding and, as such, generate both stimulatory and inhibitory intracellular platelet signals. Understanding these mechanisms may provide novel methods for manipulating platelet function in vivo. Secondly, will be further analysis on the role of the GP Ib–IX–V complex in megakaryocytopoiesis. The mechanisms that control platelet release from the mature megakaryocyte are still poorly defined. The observation that lack of a GP Ib–IX–V complex alters normal platelet release provides a starting point to use animal models to further define the mechanisms controlling this process. Thirdly, will be studies determining the three-dimensional structure of components of the GP Ib–IX–V complex. The crystal structure of the vWF domain interacting with GP Ib–IX–V has been solved, so the next logical step will be a determination of the receptor's structure or the determination of a receptor/ligand complex. Finally, the development of antithrombotics inhibiting the vWF/GP Ib–IX–V axis may have some surprising benefits over the currently used therapies. Conceptually, targeting a receptor functioning in the initial platelet response may provide a highly controlled inhibition of arterial thrombus formation with a reduced risk for spontaneous venous bleeding. When completed, these outlined studies will provide a highly detailed molecular map initiated by those astute observations of Erik von Willebrand, Jean Bernard and Jean-Pierre Soulier.

References

1 Nurden AT, Caen JP. Specific roles for platelet surface glycoproteins in platelet function. *Nature* 1975; 255: 720–722.
2 Lynch DC, Zimmerman TS, Collins CJ et al. Molecular cloning of cDNA for human von Willebrand factor: authentication by a new method. *Cell* 1985; 41: 49–56.
3 Sadler JE, Shelton-Inloes BB, Sorace JM, Harlan JM, Titani K, Davie EW. Cloning and characterization of two cDNAs coding for human von Willebrand factor. *Proc Natl Acad Sci USA* 1985; 82: 6394–6398.
4 Verweij CL, de Vries CJM, Distel B et al. Construction of cDNA coding for human von Willebrand factor using antibody probes for colony-screening and mapping of the chromosomal gene. *Nucleic Acids Res* 1985; 13: 4699–4717.
5 Vicente V, Kostel PJ, Ruggeri ZM. Isolation and functional characterization of the von Willebrand factor-binding domain located between residues His(1)-Arg(293) of the alpha-chain of glycoprotein Ib. *J Biol Chem* 1988; 263: 18473–18479.
6 De Marco L, Mazzucato M, Masotti A, Ruggeri ZM. Localization and characterization of an α-thrombin-binding site on platelet glycoprotein Ibα. *J Biol Chem* 1994; 269: 6478–6484.
7 Murata M, Ware J, Ruggeri ZM. Site-directed mutagenesis of a soluble recombinant fragment of platelet glycoprotein Ibα demonstrating negatively charged residues involved in von Willebrand factor binding. *J Biol Chem* 1991; 266: 15474–15480.
8 Roth GJ. Developing relationships: arterial platelet adhesion,

Cambridge Books Online © Cambridge University Press, 2009

glycoprotein Ib, and leucine-rich glycoproteins. *Blood* 1991; 77: 5–19.
9 Kobe B, Deisenhofer J. The leucine-rich repeat: a versatile binding motif. *Trends Biochem Sci* 1994; 19: 415–421.
10 Shen Y, Romo GM, Dong JF et al. Requirement of leucine-rich repeats of glycoprotein (GP) Ibα for shear-dependent and static binding of von Willebrand factor to the platelet membrane GP Ib–IX–V complex. *Blood* 2000; 95: 903–910.
11 Lopez JA, Andrews RK, Afshar-Kharghan V, Berndt MC. Bernard–Soulier syndrome. *Blood* 1998; 91: 4397–4418.
12 Marchese P, Saldivar E, Ware J, Ruggeri ZM. Adhesive properties of the isolated amino terminal domain of platelet glycoprotein Ibα in a flow field. *Proc Natl Acad Sci USA* 1999; 96: 7837–7842.
13 Dong J-F, Li CQ, Lopez JA. Tyrosine sulfation of the glycoprotein Ib–IX complex: Identification of sulfated residues and effect on ligand binding. *Biochemistry* J 1994; 33: 13946–13953.
14 Marchese P, Murata M, Mazzucato M, Pradella P, De Marco L, Ware J et al. Identification of three tyrosine residues of glycoprotein Ibα with distinct roles in von Willebrand factor and α-thrombin binding. *J Biol Chem* 1995; 270: 9571–9578.
15 Lopez JA, Leung B, Reynolds CC, Li CQ, Fox JEB. Efficient plasma membrane expression of a functional platelet glycoprotein Ib–IX complex requires the presence of its three subunits. *J Biol Chem* 1992; 267: 12851–12859.
16 Du X, Harris SJ, Tetaz TJ, Ginsberg MH, Berndt MC. Association of a phospholipase A2 (14–3–3 protein) with the platelet glycoprotein Ib–IX complex. *J Biol Chem* 1994; 269: 18287–18290.
17 Calverley DC, Kavanagh TJ, Roth GJ. Human signalling protein 14–3–3ζ interacts with platelet glycoprotein Ib subunits Ibα and Ibβ. *Blood* 1998; 91: 1295–1303.
18 Fox JEB. Identification of actin-binding protein as the protein linking the membrane skeleton to glycoproteins on platelet plasma membranes. *J Biol Chem* 1985; 260: 11970–11977.
19 Lopez JA, Ludwig EH, McCarthy BJ. Polymorphism of human glycoprotein Ibα results from a variable number of tandem repeats of a 13-amino acid sequence in the mucin-like macroglycopeptide region. *J Biol Chem* 1992; 267: 10055–10061.
20 Muckian C, Hillmann A, Kenny D, Shields DC. A novel variant of the platelet glycoprotein Ib alpha macroglycopeptide region lacks any copies of the 'perfect' 13 amino acid repeat. *Thromb Haemost* 2000; 83: 513–514.
21 Murata M, Furihata K, Ishida F, Russell SR, Ware J, Ruggeri ZM. Genetic and structural characterization of an amino acid dimorphism in glycoprotein Ibα involved in platelet transfusion refractoriness. *Blood* 1992; 79: 3086–3090.
22 Kuijpers RWAM, Faber NM, Cuypers HThM, Ouwehand WH, von dem Borne AEGKr. NH2-terminal globular domain of human platelet glycoprotein Ib alpha has a methionine145/threonine145 amino acid polymorphism, which is associated with HPA-2 (Ko) alloantigens. *J Clin Invest* 1992; 89: 381–384.
23 Hashimoto Y, Ware J. Identification of essential GATA and Ets binding motifs within the promoter of the platelet glycoprotein Ibα gene. *J Biol Chem* 1995; 270: 24532–24539.
24 Bastian LS, Yagi M, Chan C, Roth GJ. Analysis of the megakaryocyte glycoprotein IX promoter identifies positive and negative regulatory domains and functional GATA and Ets sites. *J Biol Chem* 1996; 271: 18554–18560.
25 Shivdasani RA, Fujiwara Y, McDevitt MA, Orkin SH. A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. *EMBO J* 1997; 16(13): 3965–3973.
26 Vyas P, Ault K, Jackson CW, Orkin SH, Shivdasani RA. Consequences of GATA-1 deficiency in megakaryocytes and platelets. *Blood* 1999; 93: 2867–2875.
27 Afshar-Kharghan V, Li CQ, Khoshnevis-Asl M, Lopez JA. Kozak sequence polymorphism of the glycoprotein (GP) Ibα gene is a major determinant of the plasma membrane levels of the platelet GP Ib- IX-V complex. *Blood* 1999; 94: 186–191.
28 Gonzalez-Conejero R, Lozano ML, Rivera J et al. Polymorphisms of platelet membrane glycoprotein Ibα associated with arterial thrombotic disease. *Blood* 1998; 92: 2771–2776.
29 Murata M, Matsubara Y, Kawano K et al. Coronary artery disease and polymorphisms in a receptor mediating shear stress-dependent platelet activation. *Circulation* 1997; 96: 3281–3286.
30 Dong JF, Gao S, Lopez JA. Synthesis, assembly, and intracellular transport of the platelet glycoprotein Ib–IX–V complex. *J Biol Chem* 1998; 273: 31449–31454.
31 Calverley DC, Yagi M, Stray SM, Roth GJ. Human platelet glycoprotein V: its role in enhancing expression of the glycoprotein Ib receptor. *Blood* 1995; 86: 1361–1367.
32 Meyer SC, Fox JE. Interaction of platelet glycoprotein V with glycoprotein Ib–IX regulates expression of the glycoproteins and binding of von Willebrand factor to glycoprotein Ib–IX in transfected cells. *J Biol Chem* 1995; 270: 14693–14699.
33 Ramakrishnan V, Reeves PS, DeGuzman F et al. Increased thrombin responsiveness in platelets from mice lacking glycoprotein V. *Proc Natl Acad Sci USA* 1999; 96: 13336–13341.
34 Kahn ML, Diacovo TG, Bainton DF, Lanza F, Coughlin SR. Glycoprotein V-deficient platelets do not exhibit a Bernard–Soulier phenotype and retain normal thrombin responsiveness. *Blood* 1999; 94: 4112–4121.
35 McGowan EB, Ding A, Detwiler TC. Correlation of thrombin-induced glycoprotein V hydrolysis and platelet activation. *J Biol Chem* 1983; 258: 11243–11248.
36 Konkle BA, Shapiro SS, Asch AS, Nachman RL. Cytokine-enhanced expression of glycoprotein Ibα in human endothelium. *J Biol Chem* 1990; 265: 19833–19838.
37 Rajagopalan V, Essex DW, Shapiro SS, Konkle BA. Tumor necrosis factor-α modulation of glycoprotein Ibα expression in human endothelial and erythroleukemia cells. *Blood* 1992; 80: 153–161.
38 Wu G, Essex DW, Meloni FJ et al. Human endothelial cells in culture and in vivo express on their surface all four components of the glycoprotein Ib/IX/V complex. *Blood* 1997; 90: 2660–2669.
39 Lian J, Guoping C, Shapiro SS, Tran L-P, Beacham DA. Glycoprotein Ibα can mediate endothelial cell migration on von Willebrand factor-containing substrata. *Exp Cell Res* 1999; 252: 114–122.
40 Perrault C, Lankhof H, Pidard D et al. Relative importance of

Cambridge Books Online © Cambridge University Press, 2009

the glycoprotein Ib-binding domain and the RGD sequence of von Willebrand factor for its interaction with endothelial cells. *Blood* 1997; 90: 2335–2344.

41 Bergmeier W, Rackebrandt K, Schroder W, Zirngibl H, Nieswandt B. Structural and functional characterization of the mouse von Willebrand factor receptor GPIb–IX with novel monoclonal antibodies. *Blood* 2000; 95: 886–893.

42 André P, Denis CV, Ware J et al. Platelets adhere to and translocate on von Willebrand factor presented by endothelium in stimulated veins. *Blood* 2000; 96: 3322–3328.

43 Ware J, Russell S, Ruggeri ZM. Generation and rescue of a murine model of platelet dysfunction: the Bernard–Soulier syndrome. *Proc Natl Acad Sci USA* 2000; 97: 2803–2808.

44 Miller JL. Platelet-type von Willebrand disease. *Thromb Haemost* 1996; 75: 865–869.

45 Celikel R, Ruggeri ZM, Varughese KI. von Willebrand factor conformation and adhesive function is modulated by an internalized water molecule. *Nature Struct Biol* 2000; 7: 881–884.

46 Ruggeri ZM, Dent JA, Saldivar E. Contribution of distinct adhesive interactions to platelet aggregation in flowing blood. *Blood* 1999; 94: 172–178.

47 Ruggeri ZM, De Marco L, Gatti L, Bader R, Montgomery RR. Platelets have more than one binding site for von Willebrand factor. *J Clin Invest* 1983; 72: 1–12.

48 Ruggeri ZM. Old concepts and new developments in the study of platelet aggregation. *J Clin Invest* 2000; 105: 699–701.

49 Ruggeri ZM. Mechanisms initiating platelet thrombus formation. *Thromb Haemost* 1997; 78: 611–616.

50 Savage B, Almus-Jacobs F, Ruggeri ZM. Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. *Cell* 1998; 94: 657–666.

51 Kulkarni S, Dopheide SM, Yap CL et al. A revised model of platelet aggregation. *J Clin Invest* 2000; 105: 783–791.

52 Tsuji S, Sugimoto M, Miyata S, Kuwahara M, Kinoshita S, Yoshioka A. Real-time analysis of mural thrombus formation in various platelet aggregation disorders: distinct shear-dependent roles of platelet receptors and adhesive proteins under flow. *Blood* 1999; 94: 968–975.

53 Fujimura Y, Kawasaki T, Titani K. Snake venom proteins modulating the interaction between von Willebrand factor platelet glycoprotein Ib. *Thromb Haemost* 1997; 76: 633–639.

54 Goto S, Ikeda Y, Saldivar E, Ruggeri ZM. Distinct mechanisms of platelet aggregation as a consequence of different shearing flow conditions. *J Clin Invest* 1998; 101: 479–486.

55 Okumura T, Hasitz M, Jamieson GA. Platelet glycocalicin. Interaction with thrombin and role as thrombin receptor of the platelet surface. *J Biol Chem* 1978; 253: 3435–3443.

56 Yamamoto N, Greco NJ, Barnard MR et al. Glycoprotein Ib (GPIb)-dependent and GPIb-independent pathways of thrombin-induced platelet activation. *Blood* 1991; 77: 1740–1748.

57 Harmon JT, Jamieson GA. The glycocalicin portion of platelet glycoprotein Ib expresses both high and moderate affinity receptor sites for thrombin. A soluble radioreceptor assay for the interaction of thrombin with platelets. *J Biol Chem* 1986; 261: 13224–13229.

58 Hayes KL, Tracy PB. The platelet high affinity binding site for thrombin mimics hirudin, modulates thrombin-induced platelet activation, and is distinct from the glycoprotein Ib–IX complex. *J Biol Chem* 1999; 274: 972–980.

59 Dong JF, Saetung G, Lopez JA. Role of glycoprotein V in the formation of the platelet high-affinity thrombin-binding site. *Blood* 1997; 89: 4355–4363.

60 Berndt MC, Ward CM, Booth WJ, Castaldi PA, Mazurov AV, Andrews RK. Identification of aspartic acid 514 through glutamic acid 542 as a glycoprotein Ib–IX complex receptor recognition sequence in von Willebrand factor. Mechanisms of modulation of von Willebrand factor by ristocetin and botrocetin. *Biochemistry* 1992; 31: 11144–11151.

61 Mazzucato M, De Marco L, Masotti A, Pradella P, Bahou WF, Ruggeri ZM. Characterization of the initial α-thrombin interaction with glycoprotein Ibα in relation to platelet activation. *J Biol Chem* 1998; 273: 1880–1887.

62 Dörmann D, Clemetson KJ, Kehrel BE. The GPIb thrombin-binding site is essential for thrombin-induced platelet procoagulant activity. *Blood* 2000; 96: 2469–2478.

63 Wang W, Shakes DC. Molecular evolution of the 14–3–3 protein family. *J Mol Evol* 1996; 43: 384–398.

64 Bodnar RJ, Gu M, Li Z, Englund GD, Du X. The cytoplasmic domain of the platelet glycoprotein Ibα is phosphorylated at serine 609. *J Biol Chem* 1999; 274: 33474–33479.

65 Gu M, Xi X, Englund GD, Berndt MC, Du X. Analysis of the roles of 14–3–3 in the platelet glycoprotein Ib–IX-mediated activation of integrin $\alpha_{IIb}\beta_3$ using a reconstituted mammalian cell expression model. *J Cell Biol* 1999; 147: 1085–1096.

66 Zaffran Y, Meyer SC, Negrescu E, Reddy KB, Fox JEB. Signalling across the platelet adhesion receptor glycoprotein Ib–IX induces $\alpha_{IIb}\beta_3$ activation both in platelets and a transfected Chinese hamster ovary cell system. *J Biol Chem* 2000; 275(22): 16779–16787.

67 Feng S, Christodoulides N, Reséndiz JC, Berndt MC, Kroll MH. Cytoplasmic domains of GpIbα and GpIbβ regulate 14–3–3ζ binding to GpIb/IX/V. *Blood* 2000; 95: 551–557.

68 Mistry N, Cranmer SL, Yuan Y et al. Cytoskeletal regulation of the platelet glycoprotein Ib/V/IX-von Willebrand factor interaction. *Blood* 2000; 96: 3480–3489.

69 Englund GD, Bodnar RJ, Li Z, Ruggeri ZM, Du X. Regulation of vWF binding to the platelet glycoprotein Ib–IX by a membrane skeleton-dependent inside-out signal. *J Biol Chem* 2000; in press.

70 Romo GM, Dong JF, Schade AJ et al. The glycoprotein Ib–IX–V complex is a platelet counterreceptor for P-selectin. *J Exp Med* 1999; 190: 803–814.

71 Takahashi R, Sekine N, Nakatake T. Influence of monoclonal antiplatelet glycoprotein antibodies on in vitro human megakaryocyte colony formation and proplatelet formation. *Blood* 1999; 93: 1951–1958.

72 Feng S, Christodoulides N, Kroll MH. The glycoprotein Ib/IX complex regulates cell proliferation. *Blood* 1999; 93: 4256–4263.

Cambridge Books Online © Cambridge University Press, 2009

13

Platelet receptors: fibrinogen

Edward F. Plow, Thomas A. Haas and Tatiana V. Byzova

Joseph J. Jacobs Center for Thrombosis and Vascular Biology

Department of Molecular Cardiology, Cleveland Clinic Foundation, Ohio, USA

Introduction and historic perspectives

Scientists have always been intrigued by the highly visible reactions. Hence, platelet aggregation and fibrin deposition, the key events in one of the most visually dramatic of biological reactions, thrombus formation, have been the subject of intensive scrutiny for many decades. Evidence for an interplay between these key components of the thrombus had already begun to evolve in the early 1960s[1–3]. Platelets in plasma derived from patients with afibrinogenemia failed to aggregate or aggregated poorly when stimulated with ADP or epinephrine, but a robust aggregation response occurred upon addition of fibrinogen to the plasma. These observations also were documented in artificially defibrinated and reconstituted plasma[4]. In the 1970s, as investigators became adept at isolating functional platelets, the role of fibrinogen in platelet aggregation was corroborated; and the requirements for an agonist, a divalent cation and fibrinogen to support the aggregation response was documented[5–7]. By the late 1970s, formal studies, utilizing approaches developed to examine hormone–receptor interactions, to measure the direct interaction of fibrinogen with platelets, were under way[6,8,9]. Thus, in the early 1980s, results from multiple laboratories had documented that platelets expressed fibrinogen receptors, capable of binding this plasma protein in a specific and saturable manner[10–15]. Furthermore, an important functional response, platelet aggregation, could be ascribed to the binding of fibrinogen to the platelet surface.

The identity of the surface molecule mediating fibrinogen binding to platelets emerged soon thereafter although its history began much earlier. In the early 1900s the Swiss physician, Glanzmann, had described a group of patients with a bleeding syndrome whose platelets failed to aggregate[16]. Nurden and Caen[17,18] were among the first to use gel electrophoresis to analyse the membrane proteins of platelets from patients defective in function and Phillips and Agin[19] demonstrated that two protein bands were missing from the membranes of platelets from patients with Glanzmann's thrombasthenia. Based upon their electrophoretic mobilities, these bands were II and III, and ultimately GPIIb and GPIIIa[20]. The failure of thrombasthenic platelets to bind fibrinogen[8,21,22], the ability of monoclonal antibodies to the GPIIb–IIIa complex to block fibrinogen binding to platelets and induce a thrombasthenic-like state[23], and the ability of isolated GPIIb–IIIa to bind fibrinogen[24–26] completed the circuit: platelet membrane glycoprotein GPIIb–IIIa served as a fibrinogen receptor on platelets and occupancy of this receptor was necessary for platelet aggregation.

Since the 1980s, the interaction of fibrinogen with platelets has remained the topic of extensive investigations. In 1980–2001, the number of citations dealing with fibrinogen and platelets totalled more than 3600 (PubMed search). The impetus for this extensive interest in this interaction comes from many directions. First, the capacity of platelets to aggregate is a central function of these cells. While we now appreciate that GPIIb–IIIa serves as a receptor for other adhesive proteins[27], and these interactions are of clear importance in platelet adhesion and aggregation, fibrinogen recognition plays a pivotal role in hemostatic and thrombotic responses of platelets. Secondly, the interaction of fibrinogen with GPIIb–IIIa served as a prototype for the analyses of other ligand interactions with platelets and for adhesive ligand interactions with receptors on many other cell types. By the mid-1980s, it was apparent that GPIIb–IIIa was a member of a growing family of structurally, immunochemically and functionally related heterodimeric adhesion receptors[28–30], which we now term the integrins[31,32]. In integrin nomenclature, GPIIb–IIIa is αIIbβ3, and this designation will be used

throughout. The ready availability of large quantities of platelets and, consequently, the capacity to isolate relatively large quantities of αIIbβ3 provided insights into integrin structure and function[33,34] that have been broadly applicable. Many of the paradigms regarding integrins, including their roles as signalling receptors, evolved from studies of αIIbβ3[35–38]. Thirdly, interference with fibrinogen binding to αIIbβ3 and its functional consequence, platelet aggregation, became the target of an intense and ultimately successful therapeutic strategy for development of antithrombotic agents to treat acute coronary syndromes and prevent the complications of cardiovascular interventions. Three such GPIIb–IIIa antagonists have been approved by the FDA in the United States, and well over 100 000 patients have been treated with these drugs[39–41].

This chapter considers the fibrinogen receptors on platelets. Therefore, predicated on the historical synopsis above, the major emphasis will be on the interaction of fibrinogen with αIIbβ3. Relevant related chapters in this volume include the contributions by Tomiyama et al. on platelet membrane proteins (Chapter 5), Ware and Ruggeri on von Willebrand factor receptors (Chapter 12) and Phillips on GPIIb–IIIa antagonists (Chapter 63). However, αIIbβ3 is not the sole mediator of fibrinogen binding to the platelet surface. Two other integrins expressed by platelets, αvβ3 and α5β1, can interact with fibrinogen. In addition, still other mechanisms exist for fibrin(ogen) to interact with the platelet surface. These αIIbβ3-independent interactions will also be considered briefly.

Platelet fibrinogen receptors: αIIbβ3

Structure of αIIbβ3

This brief consideration of the structure of αIIbβ3 emphasizes the properties that are relevant to its function as a fibrinogen receptor. Detailed reviews of the structure of αIIbβ3 can be found in the following citations[42–46] as well as in other chapters in the volume. The cellular distribution of αIIbβ3 is restricted primarily to platelets and megakaryocytes. Certain tumour cells also have been reported to express authentic αIIbβ3[47,48]. On the platelet surface, it is present at ~40 000 – 80 000 copies per cell[49,50]. This number approximates the number of fibrinogen binding sites on the surface of normal platelets[8], and it is generally accepted that each αIIbβ3 can function as a fibrinogen receptor. In addition, an intracellular pool of αIIbβ3 exists within the membranes of platelet α-granules and can become expressed at the platelet surface in association with the secretory reaction[51,52]. Thus, platelet stimulation leading to secretion can increase the number of functional fibrinogen receptors by about 30–50%[53]. Rapid shuttling of αIIbβ3 between the cell surface and an intracellular pool has been observed, and may be involved in the import of fibrinogen into α-granules[54]. Platelets from patients with Glanzmann's thrombasthenia lack functional αIIbβ3 and have reduced levels of fibrinogen within their α-granules[55]. This observation, together with the inability of human megakaryocytes to synthesize fibrinogen, as well as more direct experimental evidence indicating that platelets and megakaryocytes can internalize extracellular proteins[56–58], suggests that αIIbβ3 is involved in the transport of fibrinogen into platelet and/or megakaryocyte α-granules. Thus, α-granules contain both the ligand, fibrinogen, and the receptor, αIIbβ3; and there is evidence to suggest that these may, in part, be exported to the platelet surface as a preformed complex, capable of mediating platelet aggregation[59].

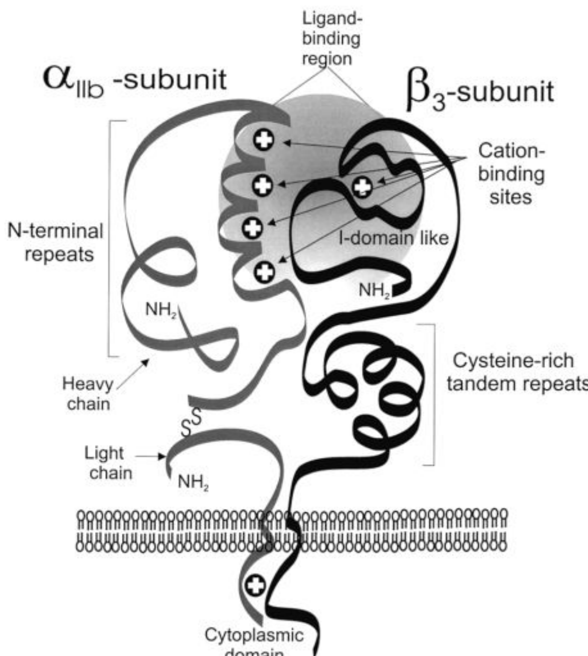


Fig. 13.1. Schematic model of integrin αIIbβ3 (adapted from ref. 40).

A schematic model of αIIbβ3 is displayed in Fig. 13.1. αIIbβ3 is a typical member of the integrin family of adhesion receptors[31,60]. As such, it is a non-covalent heterodimer composed of an alpha subunit (αIIb, GPIIb) and a beta subunit (β3,GPIIIa). Calcium is necessary to maintain αIIbβ3 as a heterodimer[61–65], explaining one of the roles of divalent cations in fibrinogen binding to this receptor. Each subunit is oriented with its N-terminus in the extracellular environment and its C-terminus within the platelet cytosol,

Cambridge Books Online © Cambridge University Press, 2009

i.e. a Type I membrane protein orientation. Each subunit is composed of a short cytoplasmic 'tail', a single transmembrane domain, and a large and glycolysated extracellular region. Based upon their electrophoretic mobility on polyacrylamide gel electrophoresis in sodium dodecylsulfate, the estimated molecular weight of αIIb is ~130 kDa and of β3 is ~95 kDa[20,66]. Mature αIIb is composed of 1108 amino acids[67,68]. Upon reduction, it separates into a heavy chain of 115 kDa and a light chain of 25 kDa. The heavy chain is entirely extracellular while the light chain contains the cytoplasmic tail and the membrane spanning region. The N-terminal aspect of the αIIb heavy chain consists of seven homologous repeating segments of ~50 amino acids, which are predicted to fold into a β-propeller-like structure[69], such as found in G-proteins. Also present in the mid-region of the αIIb heavy chain are four putative cation binding motifs of 12 amino acids, which have the spacing of oxygenated amino acids to resemble EF-hand-like structures[68], Ca^{2+} binding motifs such as is found in calmodulin. Cations are required for the binding of adhesive ligands to αIIbβ3[8,13] and are also necessary to maintain the non-covalent association between its subunits[61,62,65]. The cytoplasmic tail of αIIb is very short (21 amino acids) and contains a high-affinity cation binding site at its C-terminus[70]. Recent structural analyses by NMR suggest that this cytoplasmic tail may be composed of a short α-helix in its membrane proximal region followed by a bend which allows the C-terminus to interact with the helical region[71]. The mature β3 subunit is 762 amino acids long, and about 90% of this sequence is extracellular. A dominant feature of this subunit is a long disulfide loop, which links its N-terminal aspects to its mid-section[34,72]. The mid-segment of β3 (~90–400) and other integrin beta subunits may fold into an I-domain-like structure composed of 5–6 α-helices and a similar number of β-strands[43,73–76]. I (also referred to as A) domains are found in several integrin alpha subunits (not in αIIb) as well as several other proteins[43,77,78]. They contain a unique cation binding motif, a metal ion-dependent adhesion site (MIDAS) motif, in which non-contiguous amino acids coordinate the cation[43,73,77,79]. It is likely that the β3 subunit also contains a MIDAS motif. Not only the mid-segment but also the remainder of the C-terminus of the extracellular domain is highly conserved among integrin beta subunits. This region is composed of four cysteine-rich tandem repeats of about 40 amino acids, which renders this region resistant to proteolysis[74.80]. A single transmembrane segment is followed by a cytoplasmic tail of fewer than 50 amino acids. Within this tail are tyrosine[81,82] and threonine[83,84] phosphorylation sites and a NPLY sequence[85], which imparts a tight β-turn to the tail[86]. The extracellular regions of the αIIb and β3 subunits interact to form the extracellular domain which mediates the binding of fibrinogen and other ligands to the receptor. In addition, the cytoplasmic tails of the αIIb and β3 subunits also appear to interact with each other to form a cytoplasmic domain[70,87,88], which regulates αIIbβ3 activation, signalling and interaction of the receptor with the platelet cytoskeleton and with the signalling machinery of the cell.

Multiple approaches have been implemented to identify regions and sequences within αIIbβ3 that are involved in ligand recognition. These have included various crosslinking approaches using intact fibrinogen[89,90] as well as its recognition peptides[91–93]; identification of point mutations that inactivate the function of αIIbβ3 in thrombasthenic patients[94–100]; site-directed mutagenesis of single residues or small segments of the molecule[80,101]; and expression and functional characterization of recombinant fragments of the receptor[102,103] and its subunits[104–106]. All approaches have yielded valuable information regarding the structure–functional relationships for ligand recognition by αIIbβ3. However, an overall understanding of where and how fibrinogen binds to αIIbβ3 remains uncertain, and high resolution structures by NMR or crystallography appear to be necessary for ultimate clarification. To provide a brief synopsis of the existing information, both subunits appear to contain contact sites for ligand[107,108], and their complex is necessary for high affinity binding of fibrinogen[26,64]. The ligand binding site appears to reside in the N-terminal half of the αIIbβ3 complex. Within the αIIb subunit, the N-terminal region, residues 1–400, imparts the specificity to αIIbβ3 that distinguishes it from its sister receptor, αvβ3[109], which shares the same β3 subunit. This region contains the seven N-terminal repeats, which form the putative β-propeller, and the four EF-hand-like cation binding domains[46] (see Fig. 13.1). Various point mutations that inactivate ligand binding to integrins cluster on spatially contiguous segments of the blades of the β-propeller[69,101,110,111]. Expression of the four EF-hand-like motifs leads to a recombinant fragment with fibrinogen and cation binding properties[104], but this segment does not establish the specificity of the receptor for fibrinogen[112]. The fibrinogen γ-chain peptide also cross-links within this region[93], and peptides from within the region exhibit fibrinogen binding properties[113,114]. Within the β3 subunit, the mid-segment extending from residues 95–400 is strongly implicated in the ligand binding function of the receptor (although certain antibodies reactive with the more N-terminal aspects of the receptor can also be function blocking[115,116]). This region corresponds to the putative I-domain in the β3 subunit and contains the predicted MIDAS motif[43]. Several naturally occurring and site-directed mutations that inactivate αIIbβ3 reside within this

Cambridge Books Online © Cambridge University Press, 2009

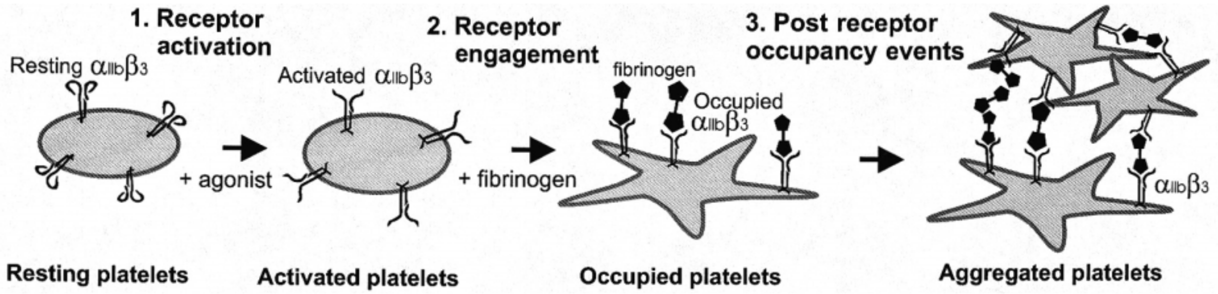


Fig. 13.2. Sequential events associated with fibrinogen binding to αIIbβ3 on platelets.

mid-segment. RGD peptides cross-link within the region of both αIIbβ3[117]and αvβ3[118]. This region also contains sequences which determine the ligand specificity of other integrins[119] and mediate recognition of monoclonal antibodies that alter the function, both activating and inhibitory, of their integrins[120], including ones to αIIbβ3[121]. Peptides containing key residues of the MIDAS domain can bind both cation and ligand in a mutually exclusive manner[122], and mutation of the cation coordinating residues within the MIDAS inhibit ligand binding[80]. Fibrinogen and RGD peptides also can bind to recombinant fragments from this β3 mid-segment[105,106]. While most attention has been focused on these two regions of the αIIb and β3 subunits, regions outside these segments can also influence receptor function. Mutations within other sites within the extracellular domain of αIIbβ3[123] and within each of the cytoplasmic tails can alter the activation states of the receptor, rendering it constitutively active or incapable of activation[124–128].

Interaction of fibrinogen with αIIbβ3: a multistep reaction

From early studies of fibrinogen binding to platelets, it became apparent that the interaction could be dissected into a series of distinct steps[13,129–131]. These steps are identified as: (i) receptor activation or induction, the process by which αIIbβ3 is transformed into a competent fibrinogen receptor; (ii) fibrinogen engagement, the reversible interaction of fibrinogen with αIIbβ3; and (iii) the postreceptor occupancy events, including the transition of fibrinogen from a reversibly to an irreversibly bound state and the intracellular signalling events initiated by αIIbβ3 occupancy. These steps are discussed separately below.

Receptor activation

Circulating platelets express αIIbβ3 at high density on their surface and are exposed to the μM concentrations of fibrinogen in blood, well in excess of the affinity of their interaction, yet the receptor does not become saturated or even minimally occupied by ligand. The reaction between fibrinogen and αIIbβ3 is tightly controlled, perhaps one of the most remarkable examples of such tight receptor regulation. This regulation is dependent upon the capacity of αIIbβ3 to transit from a resting state in which it recognizes fibrinogen with very low affinity to an activated state in which it functions as a competent receptor for the ligand. This transition must be very rapid to limit blood loss at sites of vascular injury. Thus, the first step in the sequence of events leading to engagement of αIIbβ3 by fibrinogen is receptor activation (see Fig. 13.2). This transition of αIIbβ3 from a resting to an activated state is induced by stimulation of platelets with any one of several physiological agonists. Platelet agonists that support this transition include ADP, epinephrine, thrombin, vWF, collagen, and certain arachidonic acid metabolites. Certain of these agonists (ADP, vWF and thromboxanes) are produced and released from the platelets themselves, thereby perpetuating or accelerating the activation process. Others (collagen and vWF) are the matrix proteins which mediate the initial adhesion of platelets to sites of vascular damage and, thereby, can trigger thrombus formation at these sites. While these agonists are quite distinct from one another in structure, they share the capacity to interact with receptors on the platelet surface that are capable of transducing intracellular signalling events within the cell. These ligands and their receptors are the subjects of Chapters 8–12 in this volume. Ultimately, these intracellular signalling events converge on the cytoplasmic domain of αIIbβ3 and elicit activation of the receptor. The process by which these intracellular signals are transmitted from within the cell to activate the ligand binding site within the extracellular domain is referred to as inside-out signalling[37,132]. The most proximal and specific event that activates at the cytoplasmic tails of αIIbβ3 remains to be clearly defined and could entail the association of an activator or the

Cambridge Books Online © Cambridge University Press, 2009

dissociation of an inhibitor from the cytoplasmic tails of the receptor[133]. Yeast two-hybrid screening approaches using the cytoplasmic tail of either αIIb or β3 as a bait have identified several candidate regulatory molecules[134–136] but the physiological role of any of these remains uncertain at this time. Changes in the linkages of αIIbβ3 to the platelet cytoskeleton have been strongly implicated as a possible regulator of receptor activation[86,137,138]. Phosphorylation events are common mechanisms for receptor activation. Both threonine[83,84] and tyrosine[81,82] phosphorylations occur in the cytoplasmic tail of the β3 subunit; however, these modifications appear to occur as a consequence of fibrinogen binding to αIIbβ3 and are viewed as being more involved in the transmission of outside-in signalling events rather than in receptor activation[81].

Within the context of αIIbβ3 *per se*, there are two potential mechanisms for enhancing its ligand recognition capabilities. These processes are referred to avidity or affinity modulation. Avidity modulation depends upon the clustering of receptors within the membrane so as to increase their local concentration and, thereby, drive the recognition of multivalent ligands, such as fibrinogen. Accordingly, redistribution of αIIbβ3 on the platelet membrane would be the controlling event for activation of its fibrinogen binding capacity. Redistribution of αIIbβ3 has been demonstrated by electron microscopy on the surface of adherent and spreading platelets[139–142]; and clustering of αIIbβ3 by artificial means can result in fibrinogen binding to the receptor[143]. Moreover, avidity modulation appears to be an important mechanism for integrin activation, particularly for members of the β2 subfamily[144]. Nevertheless, most data indicate that affinity modulation is the major mechanism for conversion of αIIbβ3 to a competent fibrinogen receptor. Affinity modulation depends upon an intrinsic change in the individual receptors to enhance their affinity for ligand. In the case of αIIbβ3, this is achieved by the transmission of a conformational change, initiated within the cytoplasmic domain of the receptor, across the platelet membrane to the ligand recognition site in the extracellular domain to render it competent to bind fibrinogen. Several experimental lines of evidence have been developed to demonstrate that αIIbβ3 can exist in different conformational states and can bind a soluble, monovalent ligand, indicative of affinity modulation[145,146]. Furthermore, αIIbβ3 can be isolated in a resting state[147–150], and its ligand binding function can be activated under conditions where receptor density remains constant. Nevertheless, combinations of avidity and affinity modulations may both contribute to fibrinogen binding[143].

Fibrinogen engagement

The binding of fibrinogen to activated αIIbβ3 on platelets is divalent cation-dependent and is optimally supported by millimolar calcium or magnesium concentrations[8,13]. Initially, this interaction fulfills the characteristics of true equilibrium binding. It is fully reversible and can be described by simple on and off rate constants and a dissociation constant[129,151]. The K_d of the interaction is ~300 nmol/l[8,13]. The number of fibrinogen molecules bound per platelet mirrors the number of αIIbβ3 molecules on the cell surface. Thus, 40 000 to 80 000 fibrinogen molecules are bound per platelet, and the number can exceed 100 000 if secretion has occurred to expose the granule pool of αIIbβ3[53]. However, the number of fibrinogen molecules that must be bound per platelet to support platelet aggregation is considerably less. Optimal therapy with GPIIb–IIIa antagonists seeks to attain greater than 80% inhibition of receptor occupancy, which suggests that fibrinogen binding to 20% of the available receptors is sufficient to sustain limited thrombus formation[41,152]. By virtue of its dimeric structure and the spacing of its recognition sites at distant positions in the molecule (see Fig. 14.3, fibrinogen reversibly bound to αIIbβ3 can bridge between adjacent platelets and support aggregation[153,154]. Other adhesive proteins, such as fibronectin, even though dimeric or multimeric, do not express their αIIbβ3 recognition sites in an appropriate orientation to bridge between platelets[155] and, therefore, do not support aggregation. However, the evidence that single fibrinogen molecules actually bridge between platelets linking them together in a thrombus is less than compelling. For example, fibrinogen can be bound to αIIbβ3 at high density but still fails to support aggregation[156,157]. Such observations suggest that postreceptor occupancy events are necessary to elicit an aggregation response.

Postreceptor occupancy events

The postreceptor occupancy events refer to a complex series of reactions that occur following the initial reversible binding of fibrinogen to αIIbβ3. As noted above, these events may control consequences of fibrinogen binding to the receptor as fundamental as platelet aggregation itself. The first of these postreceptor occupancy events to be identified was the transition of fibrinogen from a reversibly to an irreversibly bound state, i.e. the bound fibrinogen becomes essentially non-dissociable[13,129,130,158]. Reagents that interfere with the initial binding of fibrinogen to αIIbβ3, such as EDTA or metabolic inhibitors, fail to dissociate the ligand from the platelet[159]. Changes in the access of bound fibrinogen to proteases and to certain antibodies also occur as a consequence of the stabilization reaction[160].

Cambridge Books Online © Cambridge University Press, 2009

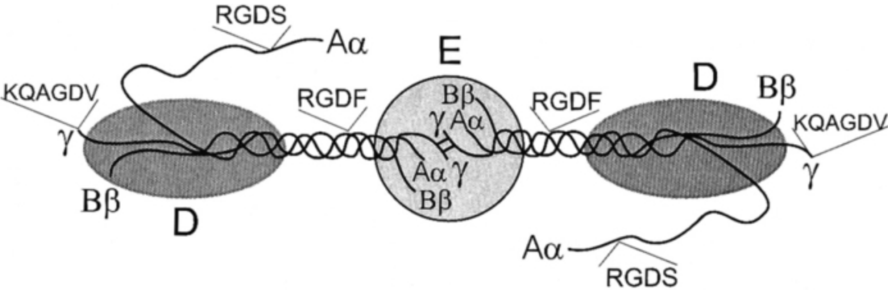


Fig. 13.3. Schematic model of fibrinogen depicting its D and E domainal organization and its constituent chains. The sequences defining the recognition specificity of αIIbβ3 on platelets are: KQAGDV at the C-terminus (406–411) of the γ-chain, RGDS at Aα572–575 and RGDF at Aα95–98.

The functional consequence of this transition is the stabilization of platelet aggregates such that they become poorly dissociable[13]. Two prevailing mechanisms have been evoked to explain the basis for irreversible fibrinogen binding. One possibility is that irreversibly bound fibrinogen remains associated with the platelet surface. For example, fibrinogen may bind to αIIbβ3 initially via its γ-chain sequence, but then may develop additional contacts, such as via the RGD or still other sequences, with the receptor, or the ligand–receptor complex becomes associated with the cytoskeleton, which stabilizes the interaction. There is support for this latter possibility[161]. The second possibility is that the bound fibrinogen becomes internalized into a distinct compartment within the platelet. Internalization of ligands bound to αIIbβ3 has been demonstrated by multiple approaches[54,162] and provides a ready explanation for the observed changes in the accessibility of bound fibrinogen to antibody and protease probes. Combinations of both mechanisms also may be operational.

A second category of postreceptor occupancy events is the induction of the LIBS and RIBS epitopes, acronyms for ligand induced binding sites and receptor induced binding sites[163]. These are epitopes recognized by monoclonal antibodies that react preferentially with the occupied receptor (LIBS) or the bound ligand (RIBS). LIBS antibodies that recognize αIIbβ3 react poorly with the resting or activated receptor, but are expressed by αIIbβ3 when it is occupied by fibrinogen and other ligands[164–168]. There are multiple LIBS within αIIbβ3, and the epitopes reside in both the αIIb and β3 subunits[169]. Certain, but not all of the LIBS, are induced by the γ-chain and RGD ligand peptides. Still other LIBS antibodies react weakly but sufficiently with resting αIIbβ3 to induce activation of the receptor[167]. Other LIBS alter functional responses of occupied αIIbβ3, including its mediation of clot retraction[165]. Antibodies with LIBS recognition properties can occur naturally and can lead to thrombocytopenia when GPIIb–IIIa antagonists are administered to some individuals[170]. RIBS are epitopes that are induced in the ligand upon its binding to the receptor[163,171–173]. Thus, there are specific monoclonal antibodies that react with fibrinogen when it is bound to αIIbβ3 but not when it is in free solution. Fibrinogen–RIBS can be induced by other modifications of the molecule, such as its deposition on surfaces or its proteolytic degradation[171,173]. Together, the induction of LIBS and RIBS indicate the dynamic nature of the postreceptor occupancy events with the induction of multiple and long-range conformational changes in both fibrinogen and αIIbβ3[174].

Additional postreceptor occupancy events include the induction of an extensive array of outside-in signalling responses. Rapid phosphorylation and activation of specific tyrosine and serine/threonine kinases, activation of specific phosphatases, recruitment of adaptor proteins, cytoskeletal rearrangements, phospholipase activation and hydrolysis of phosphoinositides are among the responses that are elicited upon fibrinogen binding to αIIbβ3[38,175,176]. Some of these outside-in signals are evoked directly by receptor occupancy, but many depend upon aggregation of the platelets. The characterization of these outside-in signalling events in platelets has provided valuable insights into the nature and regulation of integrin signalling in general. Moreover, these down-stream signalling events appear to contribute to platelet function as well. One obvious justification of this statement is mechanism of clot retraction. This contraction stabilizes the thrombus and renders it more resistant to lysis. The force for clot retraction depends upon the rearrangement of the actin cytoskeleton, which presumably 'pulls' on the cytoplasmic domain of αIIbβ3 and, thereby, on the fibrin bound to the receptor. This contractile pull on fibrin between platelets results in retraction of the thrombus. However, clot retraction is mechanistically much more complicated. Since platelets from patients with Glanzmann's thrombasthenia

Cambridge Books Online © Cambridge University Press, 2009

do not support clot retraction[177], αIIbβ3 is required for this response. Nevertheless, there is evidence that sites within fibrinogen, in addition to those known to mediate its binding to αIIbβ3, are required for clot retraction[178]. The possibility that other fibrin(ogen) receptors may participate in clot retraction is suggested from the capacity of other cell types which lack αIIbβ3 to mediate this response[179,180]. Additional evidence of the importance of these downstream events in platelet functions comes from studies of transgenic mice expressing αIIbβ3 in which the two tyrosine residues that are targets for phosphorylation in the β3 cytoplasmic tail were mutated to phenylalanines. Although this phosphorylation is detected as a postreceptor occupancy event dependent upon platelet aggregation, the aggregation response of the transgenic mouse platelets shows abnormalities[181].

Recognition of fibrinogen by αIIbβ3

Soon after the initial characterization of fibrinogen binding to αIIbβ3, efforts were initiated to identify the sites within the ligand that were recognized by the receptor. This endeavour represented a major challenge as fibrinogen is a molecule of 340 kDa and is composed of three pairs of polypeptide chains (see Fig. 13.3). Classical biochemical strategies, including proteolytic and chemical degradation and synthetic peptide approaches, were applied[153,182–185]. Ultimately, two synthetic peptides corresponding to two discrete amino acid sequences were obtained which defined the recognition specificity of αIIbβ3 for fibrinogen as well as for other adhesive ligands of the receptor[186]. The first of these peptide sets corresponds to the extreme C-terminus of the constituent γ-chain of fibrinogen[153,183], and KQAGDV was the minimal active sequence[187]. This sequence, often referred to as the γ-chain peptide, is relatively specific for αIIbβ3 and reacts considerably less well with other platelet integrin fibrinogen receptors, αvβ3 and α5β1. The second peptide, referred to as the RGD peptide, has Arg–Gly–Asp–X as its minimal sequence. RGD sequences reside at two sets of sites in the Aα-chain of fibrinogen. RGD sequences also are present within and mediate the interaction of several of the other adhesive ligands for αIIbβ3[188,189] and define the recognition specificity of several other integrins for their ligands, including αvβ3 and α5β1[190,191]. While both sequences were identified through a systematic dissection of fibrinogen, the adhesive properties of the RGD peptide had been previously identified within fibronectin[192] and had been shown to inhibit binding of this ligand[188] as well as fibrinogen and vonWillebrand factor to αIIbβ3[184]. Both peptide sets are effective inhibitors of fibrinogen binding to αIIbβ3 and platelet aggregation, and both bind directly to αIIbβ3[187,193]. This latter characteristic distinguishes these recognition peptides from other peptides derived from fibrinogen, which also inhibit platelet aggregation but do so by binding to fibrinogen[194,195]. However, of the RGD and the γ-chain sequences, it is only the latter that is essential for fibrinogen binding to αIIbβ3. Mutation of the γ-chain sequence, but not the RGD sequences, blocks fibrinogen binding to αIIbβ3 in vitro[196,197] and in vivo[198]. Secondary roles of the RGD or other sequences in mediating or stabilizing fibrinogen-αIIbβ3 interactions have been suggested, but unequivocal evidence for such a role has not been developed. Current evidence suggests that the inhibitory activity of RGD peptides may be mediated by an allosteric mechanism, i.e. they bind to αIIbβ3 and alter the conformation of the receptor such that recognition of the γ-chain sequence is blunted[199]. Among the evidence to support this interpretation are data indicating the RGD and γ-chain peptides can bind to distinct sites in αIIbβ3[106]. Nevertheless, RGD peptides have proven to be extremely valuable in defining the recognition specificity of αIIbβ3 and other integrins and in the development of GPIIb–IIIa antagonists. Several of the oral antagonists under development and the FDA approved parenteral drug, tirofiban, have used the RGD sequence as a starting point in their design[200–202].

Other platelet fibrinogen receptors

Other integrins

Fibrinogen is known to be a ligand for two members of the integrin family in addition to αIIbβ3, which are expressed on platelets. α5β1 is classically referred to as the fibronectin receptor[203–205]. About 1000 to 4000 copies of α5β1 are expressed per platelet[206]. On resting platelets, under conditions where αIIbβ3 does not contribute to binding, a low level of fibrinogen binding can be detected, which is inhibited by antibodies to α5β1[207]. This receptor recognizes the RGD sequence in the C-terminal region of the Aα chain, and its affinity for fibrinogen appears to be similar to that of αIIbβ3[207]. A role of α5β1 in adhesion of unactivated platelets to fibrin(ogen) can be envisioned but has not been demonstrated.

αvβ3, classically referred to as the vitronectin receptor[208], also binds fibrinogen employing a RGD recognition specificity[28,209–211]. αvβ3 may recognize the RGD sequence in the C-terminal aspects of the Aα chain[212,213] or a sequence unrelated to RGD[214,215]. This receptor interacts poorly with the C-terminal γ-chain sequence[213]. Thus, its

Cambridge Books Online © Cambridge University Press, 2009

recognition specificity for fibrinogen is related to but distinct from αIIbβ3. Also distinct is the divalent cation requirements for fibrinogen binding. Calcium suppresses fibrinogen binding to αvβ3 but not to αIIbβ3[216,217]. αvβ3 is present on platelets but only at a low copy number; estimates range from 200 to 500 copies of αvβ3 per platelet[210,218]. In patients with Glanzmann's thrombasthenia, the defect may reside in the αIIb or the β3 genes. In the latter case, αvβ3 is not expressed on platelets or other cells. The hemostatic abnormalities in these two classes of thrombasthenics are similar suggesting that αvβ3 plays a limited physiological role. This receptor may play a much more prominent role in pathogenesis. In tumour growth, metastasis and angiogenesis, αvβ3 is believed to play a central function[219–221]. Fibrin[222] and platelets[223] play crucial roles in tumorigenesis, and recognition of fibrin by αvβ3 may be involved in mediating their interactions with tumour cells. The importance of αvβ3 on platelets is more likely to depend upon its recognition of ligands that are not recognized by αIIbβ3. Osteopontin appears to represent such a ligand[224].

Still other fibrinogen receptors?

Thrombospondin I (TSP-I) is a major platelet α-granule protein[225,226]. Upon secretion, a portion of the released TSP-1 associates with the platelet surface[226–228]. Several candidate TSP-1 receptors have been identified on platelets including αIIbβ3[228], αvβ3[211,229], CD36 (GPIV)[230–232] and surface glycoaminoglycans[233]. Relevant to this chapter is the fact that TSP-I binds fibrinogen with high (nM^{-1}) affinity[234,235]. Thus, fibrinogen could bridge between TSP-1 molecules bound to adjacent platelets and mediate their aggregation[236].

While fibrinogen has provided a convenient ligand for analysis of binding functions, fibrin may be the more physiologically relevant ligand for the platelet. Fibrin and fibrinogen are not only structurally but also conformationally distinct. However, the insolubility and heterogeneity of fibrin has made detailed analyses of its interaction with platelets difficult. Nevertheless, some studies have been published and have suggested that fibrin interacts with αIIbβ3 with high affinity[237–239]. Fibrin may interact with additional sites in αIIbβ3 other than fibrinogen[240]. There also is evidence for αIIbβ3-independent interactions of fibrin with platelets[241]. For example, polymerizing fibrin can interact with thrombasthenic platelets[242]. Such observations suggest potentially unique interaction of fibrin(ogen) derivatives with platelets. The importance of these alternative mechanisms of interaction of fibrin(ogen) with platelets is conjectural at this time.

References

1 Caen J, Inceman S. Considerations sur l'allongement du temps de saignement dans l'afibrinogenomie congenitale. *Nouv Rev Fr Hemat* 1963; 3: 614–615.

2 Inceman S, Caen J, Bernard J. Aggregation, adhesion, and viscous metamorphosis of platelets in congenital fibrinogen deficiencies. *J Lab Clin Med* 1966; 68: 21–32.

3 Gugler E, Luscher EF. Platelet function in congenital afibrinogenemia. *Thromb Diath Haemorrh* 1965; 14: 361–373.

4 Solum NO, Stormorken H. Influence of fibrinogen on the aggregation of washed human blood platelets induced by adenosine diphosphate, thrombin, collagen and adrenaline. *Scand J Clin Lab Invest* (Suppl.17) 1965; 84: 170–182.

5 Mustard JF, Perry DW, Ardlie NG, Packham MA. Preparation of suspensions of washed platelets from humans. *Br J Haematol* 1972; 22: 193–204.

6 Mustard JF, Packham MA, Kinlough-Rathbone RL, Perry DW, Regoeczi E. Fibrinogen and ADP-induced platelet aggregation. *Blood* 1978; 52: 453–465.

7 Niewiarowski S, Budzynski AZ, Lipinski B. Significance of the intact polypeptide chains of human fibrinogen in ADP-induced platelet aggregation. *Blood* 1977; 49: 635–644.

8 Bennett JS, Vilaire G. Exposure of platelet fibrinogen receptors by ADP and epinephrine. *J Clin Invest* 1979; 64: 1393–1401.

9 Marguerie GA, Plow EF, Edgington TS. Human platelets possess an inducible and saturable receptor specific for fibrinogen. *J Biol Chem* 1979; 254: 5357–5363.

10 Peerschke EI, Zucker MB, Grant RA, Egan JJ, Johnson MJ. Correlation between fibrinogen binding to human platelets and platelet aggregability. *Blood* 1980; 55: 841–847.

11 Peerschke EI, Zucker MB. Relationship of ADP-induced fibrinogen binding to platelet shape change and aggregation elucidated by use of cholchicine and cytochalasin B. *Thromb Haemost* 1980; 43: 58–60.

12 Peerschke EI, Zucker MB. Fibrinogen receptor exposure and aggregation of human blood platelets produced by ADP and chilling. *Blood* 1981; 57: 663–670.

13 Marguerie GA, Edgington TS, Plow EF. Interaction of fibrinogen with its platelet receptor as part of a multistep reaction in ADP-induced platelet aggregation. *J Biol Chem* 1980; 255: 154–161.

14 Hawiger J, Parkinson S, Timmons S. Prostacyclin inhibits mobilisation of fibrinogen-binding sites on human ADP- and thrombin-treated platelets. *Nature* 1980; 283: 195–197.

15 Niewiarowski S, Budzynski AZ, Morinelli TA, Brudzynski TM, Stewart GJ. Exposure of fibrinogen receptor on human platelets by proteolytic enzymes. *J Biol Chem* 1981; 256: 917–925.

16 Glanzmann E. Hereditare haemorrhagische Thrombasthenie: ein beitrag zur patholigie der blutplattchen. *Jahrb Kinderheilk* 1918; 88: 113–141.

17 Nurden AT, Caen JP. Specific roles for platelet surface glycoproteins in platelet function. *Nature* 1975; 255: 720–722.

18 Nurden AT, Caen JP. An abnormal glycoprotein pattern in

Cambridge Books Online © Cambridge University Press, 2009

three cases in Glanzmann's thrombasthenia. *Br J Haematol* 1974; 28: 253–260.

19 Phillips DR, Agin PP. Platelet membrane defects in Glanzmann's thrombasthenia. Evidence for decreased amounts of two major glycoproteins. *J Clin Invest* 1977; 60: 535–545.

20 Phillips DR, Agin PP. Platelet plasma membrane glycoproteins. Evidence for the presence of nonequivalent disulfide bonds using nonreduced-reduced two dimensional gel electrophoresis. *J Biol Chem* 1977; 252: 2121–2126.

21 Mustard JF, Kinlough-Rathbone RL, Packham MA, Perry DW, Harfenist EJ, Pai KRM. Comparison of fibrinogen association with normal and thrombasthenic platelets on exposure to ADP or chymotrypsin. *Blood* 1979; 54: 987–993.

22 Coller BS. Interaction of normal, thrombasthenic, and Bernard–Soulier platelets with immobilized fibrinogen: Defective platelet-fibrinogen interaction in thrombasthenia. *Blood* 1980; 55: 169–178.

23 Coller BS, Peerschke EI, Scudder LE, Sullivan CA. A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins IIb and/or IIIa. *J Clin Invest* 1983; 72: 325–338.

24 Leung LL, Kinoshita T, Nachman RL. Isolation, purification, and partial characterization of platelet membrane glycoproteins IIb and IIIa. *J Biol Chem* 1981; 256: 1994.

25 Nachman RL, Leung LLK. Complex formation of platelet membrane glycoproteins IIb and IIIa with fibrinogen. *J Clin Invest* 1982; 69: 263–269.

26 Parise LV, Phillips DR. Reconstitution of the purified platelet fibrinogen receptor. Fibrinogen binding properties of the glycoprotein IIb–IIIa complex. *J Biol Chem* 1985; 260: 10698–10707.

27 Plow EF, Ginsberg MH, Marguerie GA. Expression and function of adhesive proteins on the platelet surface. In DR Phillips, Shuman MA, *Biochemistry of Platelets* eds. 1986; Orlando, FL: Academic Press, 226–256.

28 Pytela R, Pierschbacher MD, Ginsberg MH, Plow EF, Ruoslahti E. Platelet membrane glycoprotein IIb/IIIa: Member of a family of Arg–Gly–Asp-specific adhesion receptors. *Science* 1986; 231: 1559–1562.

29 Plow EF, Loftus JC, Levin EG et al. Immunologic relationship between platelet membrane glycoprotein GPIIb/IIIa and cell surface molecules expressed by a variety of cells. *Proc Natl Acad Sci USA* 1986; 83: 6002–6006.

30 Charo IF, Fitzgerald LA, Steiner B, Rall SC, Jr, Bekeart LS, Phillips DR. Platelet glycoproteins IIb and IIIa: evidence for a family of immunologically and structurally related glycoproteins in mammalian cells. *Proc Natl Acad Sci USA* 1986; 83: 8351–8355.

31 Hynes RO. Integrins: a family of cell surface receptors. *Cell* 1987; 48: 549–550.

32 Hynes RO. Integrins: versatility, modulation, and signalling in cell adhesion. *Cell* 1992; 69: 11–25.

33 Calvete JJ, Henschen A, Gonzalez-Rodriguez J. Complete localization of the intrachain disulphide bonds and the N-glycosylation points in the alpha-subunit of human platelet glycoprotein IIb. *Biochem J* 1989; 261: 561–568.

34 Calvete JJ, Henschen A, Gonzalez-Rodriguez J. Assignment of disulphide bonds in human platelet GPIIIa. A disulphide pattern for the β-subunits of the integrin family. *Biochem J* 1991; 274: 63–71.

35 Schwartz MA, Schaller MD, Ginsberg MH. Integrins: emerging paradigms of signal transduction. *Annu Rev Cell Biol* 1995; 11: 549–599.

36 Shattil SJ, Ginsberg MH, Brugge JS. Adhesive signalling in platelets. *Curr Opin Cell Biol* 1994; 6: 695–704.

37 Ginsberg MH, Du X, Plow EF. Inside-out integrin signalling. *Curr Opin Cell Biol* 1992; 4: 766.

38 Shattil SJ, Kashiwagi H, Pampori N. Integrin signalling: the platelet paradigm. *Blood* 1998; 91: 2645–2657.

39 Coller BS. GPIIb–IIIa antagonists: pathophysiologic and therapeutic insights from studies of c7E3 Fab. *Thromb Haemost* 1997; 78: 730–735.

40 Topol EJ, Byzova TV, Plow EF. Platelet GPIIb–IIIa blockers. *Lancet* 1999; 353: 227–231.

41 Scarborough RM, Kleiman NS, Phillips DR. Platelet glycoprotein IIb/IIIa antagonists. What are the relevant issues concerning their pharmacology and clinical use? *Circulation* 1999; 100: 437–444.

42 Phillips DR, Charo IF, Parise LV, Fitzgerald LA. The platelet membrane glycoprotein IIb–IIIa complex. *Blood* 1988; 71: 831–843.

43 Loftus JC, Liddington RC. Perspectives series: cell adhesion in vascular biology. New insights into integrin-ligand interaction. *J Clin Invest* 1997; 99: 2302–2306.

44 Plow EF, Shattil SJ. Integrin αIIbβ3 expression. In RW Colman, J Hirsh, VJ Marder, AW Clowes, JN George, eds. *Hemostasis and thrombosis: basic principles and clinical practice.* 2001; Lippincott Philadelphia: Williams & Wilkins, 479–491.

45 Calvete JJ. Platelet integrin GPIIb/IIIa: Structure–function correlations. An update and lessons from other integrins. *Proc Soc Exp Biol Med* 1999; 222: 29–38.

46 Plow EF, Haas TA, Zhang L, Loftus J, Smith JW. Ligand binding to integrins. *J Biol Chem* 2000; 275: 21785–21788.

47 Honn KV, Chen YQ, Timar J et al. $\alpha_{IIb}\beta_3$ integrin expression and function in subpopulations of murine tumors. *Exp Cell Res* 1992; 201: 23–32.

48 Trikha M, Timar J, Lundy SK et al. Human prostate carcinoma cells express functional $\alpha_{IIb}\beta_3$ integrin. *Cancer Res* 1996; 56: 5071–5078.

49 Newman PJ, Allen RW, Kahn RA, Kunicki TJ. Quantitation of membrane glycoprotein IIIa on intact platelets using the monoclonal antibody, AP-3. *Blood* 1985; 65: 227–232.

50 Wagner CL, Mascelli MA, Neblock DS, Weisman HF, Coller BS, Jordan RE. Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets. *Blood* 1996; 88: 907–914.

51 Woods VL, Wolff LE, Keller DM. Resting platelets contain a

Cambridge Books Online © Cambridge University Press, 2009

substantial centrally located pool of glycoprotein IIb–IIIa complex which may be accessible to some but not other extracellular proteins. *J Biol Chem* 1986; 261: 15242–15251.
52 Wencel-Drake JD, Plow EF, Kunicki TJ, Woods VL, Keller DM, Ginsberg MH. Localization of internal pools of membrane glycoproteins involved in platelet adhesive responses. *Am J Pathol* 1986; 124: 324–334.
53 Niiya K, Hodson E, Bader R et al. Increased surface expression of the membrane glycoprotein IIb/IIIa complex induced by platelet activation. Relationship to the binding of fibrinogen and platelet aggregation. *Blood* 1987; 70: 475–483.
54 Wencel-Drake JD, Frelinger AL, III Dieter MG, Lam SCT. Arg–gly–asp-dependent occupancy of GPIIb–IIIa by applaggin: evidence for internalization and cycling of a platelet integrin. *Blood* 1993; 81: 62–69.
55 Lee H, Nurden AT, Thomaidis A, Caen JP. Relationship between fibrinogen binding and the platelet glycoprotein deficiencies in Glanzmann's thrombasthenia type I and type II. *Br J Haematol* 1981; 48: 47–57.
56 Handagama PJ, Shuman MA, Bainton DF. Incorporation of intravenously injected albumin, immunoglobulin G, and fibrinogen in guinea pig megakaryocyte granules. *J Clin Invest* 1989; 84: 73–82.
57 Handagama PJ, George JN, Shuman MA, McEver RP, Bainton DF. Incorporation of a circulating protein into megakaryocyte and platelet granules. *Proc Natl Acad Sci USA* 1987; 84: 861–865.
58 Handagama PJ, Shuman MA, Bainton DF. *In vivo* defibrination results in markedly decreased amounts of fibrinogen in rat megakaryocytes and platelets. *Am J Pathol* 1990; 137: 1393–1399.
59 Courtois G, Ryckwaert J-J, Ginsberg MH, Woods VL, Jr, Plow EF, Marguerie GA. Expression of intracellular fibrinogen on the surface of stimulated platelets. *Eur J Biochem* 1986; 159: 61–67.
60 Ginsberg MH, Loftus JC, Plow EF. Cytoadhesins, integrins and platelets. *Thromb Haemost* 1988; 59: 1–6.
61 Kunicki TJ, Pidard D Rosa J-P, Nurden AT. The formation of Ca++ dependent complexes of platelet membranes glycoprotein IIb and IIIa in solution as determined by crossed/immunoelectrophoresis. *Blood* 1981; 58: 268–278.
62 Jennings LK, Phillips DR. Purification of glycoproteins IIb and III from human platelet plasma membranes and characterization of a calcium-dependent glycoprotein IIb–III complex. *J Biol Chem* 1982; 257: 10458–10466.
63 Fujimura K, Phillips DR. Calcium cation regulation of glycoprotein IIb–IIIa complex formation in platelet plasma membranes. *J Biol Chem* 1983; 258: 10247–10252.
64 Brass LF, Shattil SJ, Kunicki TJ, Bennett JS. Effect of calcium on the stability of the platelet membrane glycoprotein IIb-IIIa complex. *J Biol Chem* 1985; 260: 7875–7881.
65 Pidard D, Didry D, Kunicki TJ, Nurden AT. Temperature-dependent effects of EDTA on the membrane glycoprotein IIb–IIIa complex and platelet aggregability. *Blood* 1986; 67: 604–611.
66 Numano F, Kishi Y, Ashikaga T, Hata A, Makita T, Watanabe R. What effect does controlling platelets have on atherosclerosis. *Ann N Y Acad Sci* 1995; 748: 383–393.
67 Poncz M, Eisman R, Heidenreich R et al. Structure of the platelet membrane glycoprotein IIb. Homology to the alpha subunits of the vitronectin and fibronectin membrane receptors. *J Biol Chem* 1987; 262: 8476–8482.
68 Fitzgerald LA, Poncz M, Steiner B, Rall SC, Jr, Bennett JS, Phillips DR. Comparison of cDNA-derived protein sequences of the human fibronectin and vitronectin receptor alpha-subunits and platelet glycoprotein IIb. *Biochemistry* 1987; 26: 8158–8165.
69 Springer TA. Folding of the N-terminal ligand-binding region of integrin α-subunits into a β-propeller domain. *Proc Natl Acad Sci USA* 1997; 94: 65–72.
70 Haas TA, Plow EF. The cytoplasmic domain of $\alpha_{IIb}\beta_3$: a ternary complex of the integrin α and β subunits and a divalent cation. *J Biol Chem* 1996; 271: 6017–6026.
71 Vinogradova O, Haas T, Plow EF, Qin J. A structural basis for integrin activation by the cytoplasmic tail of the α_{IIb} subunit. *Proc Natl Acad Sci USA* 2000; 97: 1450–1455.
72 Beer J, Coller BS. Evidence that platelet glycoprotein IIIa has a large disulfide-bonded loop that is susceptible to proteolytic cleavage. *J Biol Chem* 1989; 264: 17564–17573.
73 Lee J-O, Rieu P, Arnaout MA, Liddington R. Crystal structure of the A domain from the α subunit of integrin CR3 (CD11b/CD18). *Cell* 1995; 80: 631–638.
74 Tozer EC, Liddington RC, Sutcliffe MJ, Smeeton AH, Loftus JC. Ligand binding to integrin $\alpha_{IIb}\beta_3$ is dependent on a MIDAS-like domain in the β_3 subunit. *J Biol Chem* 1996; 271: 21978–21984.
75 Puzon-McLaughlin W, Takada Y. Critical residues for ligand binding in an I domain-like structure of the integrin β_1 subunit. *J Biol Chem* 1996; 271: 20438–20443.
76 Huang C, Zang Q, Takagi J, Springer TA. Structural and functional studies with antibodies to the integrin β2 subunit. A model for the I-like domain. *J Biol Chem* 2000; 275: 21514–21524.
77 Leitinger B, Hogg N. Integrin I domains and their function. *Biochem Soc Trans* 1999; 27: 826–832.
78 Shimaoka M, Shifman JM, Jing H, Takagi L, Mayo SL, Springer TA. Computational design of an integrin I domain stabilized in the open high affinity conformation. *Nat Struct Biol* 2000; 7: 674–678.
79 Qu A, Leahy DJ. Crystal structure of the I-domain from the CD11a/CD18 (LFA-1, $\alpha_L\beta2$) integrin. *Proc Natl Acad Sci USA* 1995; 92: 10277–10281.
80 Bajt ML, Loftus JC. Mutation of a ligand binding domain of β_3 integrin: integral role of oxygenated residues in $\alpha_{IIb}\beta_3$ receptor function. *J Biol Chem* 1994; 269: 20913–20919.
81 Law DA, Nannizzi-Alaimo L, Phillips DR. Outside-in integrin signal transduction. $\alpha_{IIb}\beta_3$-(GPIIb–IIIa) tyrosine phosphorylation induced by platelet aggregation. *J Biol Chem* 1996; 271: 10811–10815.
82 Jenkins AL, Nannizzi-Alaimo L, Silver D, Sellers JR, Ginsburg

Cambridge Books Online © Cambridge University Press, 2009

MH, Law DA, Phillips DR. Tyrosine phosphorylation of the β_3 cytoplasmic domain mediates integrin-cytoskeletal interactions. *J Biol Chem* 1998; 273: 13878–13885.

83 Lerea KM, Cordero KP, Sakariassen KS, Kirk RI, Fried VA. Phosphorylation sites in the integrin β_3 cytoplasmic domain in intact platelets. *J Biol Chem* 1999; 274: 1914–1919.

84 Kirk RI, Sanderson MR, Lerea KM. Threonine phosphorylation of the β_3 integrin cytoplasmic tail, at a site recognized by PDK1 and Akt/PKB in vitro, regulates Shc binding. *J Biol Chem* 2000; 275: 30901–30906.

85 O'Toole TE, Ylanne J, Culley BM. Regulation of integrin affinity states through an NP*X*Y motif in the β subunit cytoplasmic domain. *J Biol Chem* 1995; 270: 8553–8558.

86 Ulmer TS, Yaspan B, Ginsberg MH, Campbell ID. NMR analysis of structure and dynamics of the cytosolic tails of integrin alphaIIbbeta3 in aqueous solution. *Biochemistry* 2001; 40: 7498–7508.

87 Muir TW, Williams MJ, Ginsberg MH, Kent SBH. Design and chemical synthesis of a neoprotein structural model for the cytoplasmic domain of a multisubunit cell- surface receptor: Integrin $\alpha_{IIb}\beta_3$ (platelet GPIIb–IIIa). *Biochemistry* 1994; 33: 7701–7708.

88 Vallar L, Melchior C, Plancon S et al. Divalent cations differentially regulate integrin α_{IIb} cytoplasmic tail binding to β_3 and to calcium- and integrin-binding protein. *J Biol Chem* 1999; 274: 17257–17266.

89 Bennett JS, Vilaire G, Cines DB. Identification of the fibrinogen receptor on human platelets by photoaffinity labelling. *J Biol Chem* 1982; 257: 8049–8054.

90 Marguerie GA, Thomas-Maison N, Ginsberg MH, Plow EF. The platelet-fibrinogen interaction. Evidence for proximity of the A-alpha chain of fibrinogen to platelet membrane glycoproteins IIb/III. *Eur J Biochem* 1984; 139: 5–11.

91 Santoro SA, Lawing WJ, Jr. Competition for related but non-identical binding sites on the glycoprotein IIb–IIIa complex by peptides derived from platelet adhesive proteins. *Cell* 1987; 48: 867–873.

92 D'Souza SE, Ginsberg MH, Lam SCT, Plow EF. Chemical cross-linking of arginyl–glycyl–aspartic acid peptides to an adhesion receptor on platelets. *J Biol Chem* 1988; 263: 3943–3951.

93 D'Souza SE, Ginsberg MH, Burke TA, Plow EF. The ligand binding site of the platelet integrin receptor GPIIb–IIIa is proximal to the second calcium domain of its alpha subunit. *J Biol Chem* 1990; 265: 3440–3446.

94 Loftus JC, O'Toole TE, Plow EF, Glass A, Frelinger AL, Ginsberg MH. A β_3 integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. *Science* 1990; 249: 915–918.

95 Bajt ML, Ginsberg MH, Frelinger AL, III Berndt MC, Loftus JC. A spontaneous mutation of integrin $\alpha_{IIb}\beta_3$ (platelet glycoprotein IIb–IIIa) helps define a ligand binding site. *J Biol Chem* 1992; 267: 3789–3794.

96 Lanza F, Stierlé A, Fournier D et al. A new variant of Glanzmann's thrombasthenia (Strasbourg I). Platelets with functionally defective glycoprotein IIb–IIIa complexes and a glycoprotein IIIa ^{214}Arg→^{214}Trp mutation. *J Clin Invest* 1992; 89: 1995–2004.

97 Poncz M, Rifat S, Coller BS et al. Glanzmann thrombasthenia secondary to a gly273→asp mutation adjacent to the first calcium-binding domain of platelet glycoprotein IIb. *J Clin Invest* 1994; 93: 172–179.

98 Wilcox DA, Paddock CM, Lyman S, Gill JC, Newman PJ. Glanzmann thrombasthenia resulting from a single amino acid substitution between the second and third calcium-binding domains of GPIIb. Role of the GPIIb amino terminus in integrin subunit association. *J Clin Invest* 1995; 95: 1553–1560.

99 Basani RB, Brown DL, Vilaire G, Bennett JS, Poncz M. A Leu117→Trp mutation within the RGD-peptide cross-linking region of β_3 results in Glanzmann thrombasthenia by preventing $\alpha_{IIb}\beta_3$ export to the platelet surface. *Blood* 1997; 90: 3082–3088.

100 Grimaldi CM, Chen F, Wu C, Weiss HJ, Coller BS, French DL. Glycoprotein IIb Leu214Pro mutation produces Glanzmann thrombasthenia with both quantitative and qualitative abnormalities in GPIIb/IIIa. *Blood* 1998; 91: 1532–1571.

101 Kamata T, Irie A, Tokuhira M, Takada Y. Critical residues of integrin αIIb subunit for binding of $\alpha_{IIb}\beta_3$ (glycoprotein IIb–IIIa) to fibrinogen and ligand- mimetic antibodies (PAC-1, OP-G2, and LJ-CP3). *J Biol Chem* 1996; 271: 18610–18615.

102 Wippler J, Kouns WC, Schlaeger EJ, Kuhn H, Hadvary P, Steiner B. The integrin $\alpha_{IIb}\beta_3$, platelet glycoprotein IIb–IIIa, can form a functional active heterodimer complex without the cysteine-rich repeats of the β_3 subunit. *J Biol Chem* 1994; 269: 8754–8761.

103 Peterson JA, Visentin GP, Newman PJ, Aster RH. A recombinant soluble form of the integrin $\alpha_{IIb}\beta_3$ (GPIIb–IIIa) assumes an active, ligand-binding conformation and is recognized by GPIIb–IIIa specific monoclonal, allo-, auto-, and drug-dependent platelet antibodies. *Blood* 1998; 92: 2053–2063.

104 Gulino D, Boudignon C, Zhang LY, Concord E, Rabiet MJ, Marguerie G. Ca(2+)-binding properties of the platelet glycoprotein IIb ligand-interacting domain. *J Biol Chem* 1992; 267: 1001–1007.

105 Alemany M, Concord E, Garin J et al. Sequence 274–368 in the β_3-subunit of the integrin $\alpha_{IIb}\beta_3$ provides a ligand recognition and binding domain for the gamma-chain of fibrinogen that is independent of platelet activation. *Blood* 1996; 87: 592–601.

106 Cierniewski CS, Byzova T, Papierak M et al. Peptide ligands can bind to distinct sites in integrin $\alpha_{IIb}\beta_3$ and elicit different functional responses. *J Biol Chem* 1999; 274: 16923–16932.

107 Plow EF, D'Souza SE, Ginsberg MH. Ligand binding to GPIIb–IIIa: A status report. *Semin Thromb Hemost* 1992; 18: 324–332.

108 Parise LV, Steiner B, Nannizzi L, Criss AB, Phillips DR. Evidence for novel binding sites on the platelet glycoprotein IIb and IIIa subunits and immobilized fibrinogen. *Biochem J* 1993; 289: 445–451.

109 Loftus JC, Halloran CE, Ginsberg MH, Feigen LP, Zablocki JA, Smith JW. The amino-terminal one-third of α_{IIb} defines the

Cambridge Books Online © Cambridge University Press, 2009

ligand recognition specificity of integrin $\alpha_{IIb}\beta_3$. *J Biol Chem* 1996; 271: 2033–2039.

110 Irie A, Kamata T, Takada Y. Multiple loop structures critical for ligand binding of the integrin α4 subunit in the upper face of the β-propeller mode 1. *Proc Natl Acad Sci USA* 1997; 94: 7198–7203.

111 Puzon-McLaughlin W, Kamata T, Takada Y. Multiple discontinuous ligand-mimetic antibody binding sites define a ligand binding pocket in integrin $\alpha_{IIb\beta3}$. *J Biol Chem* 2000; 275: 7795–7802.

112 Gidwitz S, Lyman S, White GC, II. Expression and function of calcium binding domain chimeras of the integrins α_{IIb} and α_5. *J Biol Chem* 2000; 275: 6680–6688.

113 D'Souza SE, Ginsberg MH, Matsueda GR, Plow EF. A discrete sequence in a platelet integrin is involved in ligand recognition. *Nature* 1991; 350: 66–68.

114 Taylor DB, Gartner TK. A peptide corresponding to GPIIb$_\alpha$ 300–312 a presumptive fibrinogen gamma-chain binding site on the platelet integrin GPIIb/IIIa, inhibits the adhesion of platelets to at least four adhesive ligands. *J Biol Chem* 1992; 267: 11729–11733.

115 Calvete JJ, Arias J, Alvarez MV, Lopez MM, Henschen A, Gonzalez-Rodriguez J. Further studies on the topography of the *N*-terminal region of human platelet glycoprotein IIIa. Localization of monoclonal antibody epitopes and the putative fibrinogen-binding sites. *Biochem J* 1991; 274: 457–463.

116 Honda S, Tomiyama Y, Pelletier AJ et al. Topography of ligand-induced binding sites, including a novel cation-sensitive epitope (AP5) at the amino terminus, of the human integrin β_3 subunit. *J Biol Chem* 1995; 270: 11947–11954.

117 D'Souza SE, Ginsberg MH, Burke TA, Lam SCT, Plow EF. Localization of an Arg–Gly–Asp recognition site within an integrin adhesion receptor. *Science* 1988; 242: 91–93.

118 Smith JW, Cheresh DA. The Arg–Gly–Asp binding domain of the vitronectin receptor. *J Biol Chem* 1988; 263: 18726–18731.

119 Takagi J, Kamata T, Meredith J, Puzon-McLaughlin W, Takada Y. Changing ligand specificities of $\alpha_v\beta_1$ and $\alpha_v\beta_3$ integrins by swapping a short diverse sequence of the β subunit. *J Biol Chem* 1997; 272: 19794–19800.

120 Luque A, Gomez M, Puzon W, Takada Y, Sanchez-Madrid F, Cabanas C. Activated conformations of very late activation integrins detected by a group of antibodies (HUTS) specific for a novel regulatory region (355–425) of the common β1 chain. *J Biol Chem* 1996; 271: 11067–11075.

121 Andrieux A, Rabiet M-J, Chapel A, Concord E, Marguerie G. A highly conserved sequence of the Arg–Gly–Asp-binding domain of the integrin beta-3 subunit is sensitive to stimulation. *J Biol Chem* 1991; 266: 14202–14207.

122 D'Souza SE, Haas TA, Piotrowicz RS et al. Ligand and cation binding are dual functions of a discrete segment of the integrin beta-3 subunit: cation displacement from this site is implicated in ligand binding. *Cell* 1994; 79: 659–667.

123 Kashiwagi H, Tomiyama Y, Tadokoro S et al. A mutation in the extracellular cystein-rich repeat region of the β_3 subunit activates integrins $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$. *Blood* 1999; 93: 2559–2568.

124 Hughes PE, Diaz-Gonzalez F, Leong L et al. Breaking the integrin hinge. A defined structural constraint regulates integrin signalling. *J Biol Chem* 1996; 271: 6571–6574.

125 Hughes PE, O'Toole TE, Ylanne J, Shattil SJ, Ginsberg MH. The conserved membrane-proximal region of an integrin cytoplasmic domain specifies ligand binding affinity. *J Biol Chem* 1995; 270: 12411–12417.

126 O'Toole TE, Katagiri Y, Faull RJ et al. Integrin cytoplasmic domains mediate inside-out signal transduction. *J Cell Biol* 1994; 124: 1047–1059.

127 Chen Y-P, Djaffar I, Pidard D et al. Ser-752→Pro mutation in the cytoplasmic domain of integrin β_3 subunit and defective activation of platelet integrin $\alpha_{IIb}\beta_3$ (GPIIb–IIIa) in a variant of Glanzmann's thrombasthenia. *Proc Natl Acad Sci USA* 1992; 89: 10169–10173.

128 Leisner TM, Wencel-Drake JD, Wang W, Lam SC. Bidirectional transmembrane modulation of integrin $\alpha_{IIb}\beta_3$ conformations. *J Biol Chem* 1999; 274: 12945–12949.

129 Marguerie GA, Plow EF. Interaction of fibrinogen with its platelet receptor: Kinetics and the effect of pH and temperature. *Biochemistry* 1981; 20: 1074–1080.

130 Peerschke EI, Wainer JA. Examination of irreversible platelet fibrinogen interactions. *Am J Physiol* 1985; 248: C466–C472.

131 Muller B, Zerwes HG, Tangeman K, Peter J, Engel J. Two-step binding mechanism of fibrinogen to $\alpha_{IIb}\beta_3$ integrin reconstituted into planar lipid bilayers. *J Biol Chem* 1993; 268: 6800–6808.

132 Chen YP, O'Toole TE, Shipley T et al. 'Inside-out' signal transduction initiated by isolated integrin cytoplasmic domains. *J Biol Chem* 1994; 269: 18307–18310.

133 Liu SC, Calderwood DA, Ginsberg MH. Integrin cytoplasmic domain-binding proteins. *J Cell Sci* 2000; 113: 3563–3571.

134 Kashiwagi H, Schwartz MA, Eigenthaler M, Davis KA, Ginsberg MH, Shattil SJ. Affinity modulation of platelet integrin $\alpha_{IIb}\beta_3$ by β_3-endonexin, a selective binding partner of the β_3 integrin cytoplasmic tail. *J Cell Biol* 1997; 137: 1433–1443.

135 Naik UP, Patel PM, Parise LV. Identification of a novel calcium-binding protein that interacts with the integrin α_{IIb} cytoplasmic domain. *J Biol Chem* 1997; 272: 4651–4654.

136 Reddy KB, Gascard P, Price MG, Negrescu EV, Fox JE. Identification of an interaction between the m-band protein skelemin and beta-integrin subunits. Colocalization of a skelemin-like protein with beta1- and beta3-integrins in non-muscle cells. *J Biol Chem* 1998; 273: 35039–35047.

137 Bennett JS, Zigmond S, Vilaire G, Cunningham ME, Bednar B. The platelet cytoskeleton regulates the affinity of the integrin $\alpha_{IIb}\beta_3$ for fibrinogen. *J Biol Chem* 1999; 274: 30233–30241.

138 Calderwood DA, Zent R, Grant R, Rees DJ, Hynes RO, Ginsberg MH. The Talin head domain binds to integrin beta subunit cytoplasmic tails and regulates integrin activation. *J Biol Chem* 1999; 274: 28071–28074.

139 Loftus JC, Albrecht RM. Redistribution of the fibrinogen receptor of human platelets after surface activation. *J Cell Biol* 1984; 99: 822–829.

140 Isenberg WM, McEver R-P, Phillips DR, Shuman MA, Bainton

Cambridge Books Online © Cambridge University Press, 2009

DF. The platelet fibrinogen receptor: an immunogold-surface replica study of agonist-induced ligand binding and receptor clustering. *J Cell Biol* 1987; 104: 1655–1663.
141 Lewis JC, Hantgan RR, Stevenson SC et al. Fibrinogen and glycoprotein IIb/IIIa localization during platelet adhesion: Localization to the granulomere and at sites of platelet interaction. *Am J Pathol* 1990; 136: 239–252.
142 Olorundare OE, Simmons SR, Albrecht RM. Evidence for two mechanisms of ligand–receptor movement on surface-activated platelets. *Eur J Cell Biol* 1993; 60: 131–145.
143 Hato T, Pampori N, Shattil SJ. Complementary roles for receptor clustering and conformational change in the adhesive and signalling functions of integrin $\alpha_{IIb}\beta_3$. *J Cell Biol* 1998; 141: 1685–1695.
144 Porter JC, Hogg N. Integrins take partners: cross-talk between integrins and other membrane receptors. *Trends Cell Biol* 1998; 8: 390–396.
145 O'Toole TE, Loftus JC, Du X et al. Affinity modulation of the $\alpha_{IIb}\beta_3$ integrin (platelet GPIIb–IIIa) is an intrinsic property of the receptor. *Cell Regulation* 1990; 1: 883–893.
146 Sims PJ, Ginsberg MH, Plow EF, Shattil SJ. Effect of platelet activation on the conformation of the plasma membrane glycoprotein IIb–IIIa complex. *J Biol Chem* 1991; 266: 7345.
147 Steiner B, Cousot A, Trzeciak A, Gillessen D, Hadvary P. Ca^{2+}-dependent binding of a synthetic Arg–Gly–Asp (RGD) peptide to a single site on the purified platelet glycoprotein IIb–IIIa complex. *J Biol Chem* 1989; 264: 13102–13108.
148 Lam SCT, Forsyth J, Pierschbacher MD, Ruslahti E, Plow EF, Ginsberg MH. A synthetic peptide containing a fibronectin cell attachment sequence inhibits platelet aggregation and binds specifically and saturably to platelets. *Fed Proc* 1985; 44: 1126.
149 Fitzgerald LA, Leung B, Phillips DR. A method for purifying the platelet membrane glycoprotein IIb–IIIa complex. *Anal Biochem* 1985; 151: 169–177.
150 Du X, Plow EF, Frelinger AL, III, O'Toole TE, Loftus JC, Ginsberg MH. Ligands 'Activate' integrin $\alpha_{IIb}\beta_3$ (Platelet GPIIb–IIIa). *Cell* 1991: 65: 409–416.
151 Huber W, Hurst J, Schlatter D et al. Determination of kinetic constants for the interaction between the platelet glycoprotein IIb–IIIa and fibrinogen by means of surface plasmon resonance. *Eur J Biochem* 1995; 227: 647–656.
152 Coller BS. Blockade of platelet GPIIb/IIIa receptors as an antithrombotic strategy. *Circulation* 1995; 92: 2373–2380.
153 Kloczewiak M, Timmons S, Lukas TJ, Hawiger J. Platelet receptor recognition site on human fibrinogen. Synthesis and structure-function relationship of peptides corresponding to the carboxy-terminal segment of the gamma chain. *Biochemistry* 1984; 23: 1767–1774.
154 Gawaz,MP, Loftus JC, Bajt ML, Frojmovic MM, Plow EF, Ginsberg MH. Ligand bridging mediates integrin $\alpha_{IIIb}\beta_3$ (platelet GPIIb–IIIa) dependent homotypic and heterotypic cell–cell interactions. *J Clin Invest* 1991; 88: 1128–1134.
155 Ugarova TP, Zamarron C, Veklich Y et al. Conformational transitions in the cell binding domain of fibronectin. *Biochemistry* 1995; 34: 4457–4466.
156 Peerschke EI. Ca^{+2} mobilization and fibrinogen binding of platelets refractory to adenosine diphosphate stimulation. *J Lab Clin Med* 1985; 106: 111–122.
157 Sloan SM, Brown EB, Liu Q, Frojmovic MM. Glycoprotein IIb–IIIa-liposomes bind fibrinogen but do not undergo fibrinogen-mediated aggregation. *Platelets* 2000 11: 99–110.
158 Peerschke EI. Irreversible platelet fibrinogen interactions occur independently of fibrinogen alpha chain degradation and are not mediated by intact platelet membrane glycoprotein IIb–IIIa complexes. *J Lab Clin Med* 1988; 111: 84.
159 Peerschke EI. Stabilization of platelet-fibrinogen interactions: modulation by divalent cations. *J Lab Clin Med* 1993; 121: 135–141.
160 Peerschke EI. Decreased accessibility of platelet-bound fibrinogen to antibody and enzyme probes. *Blood* 1989; 74: 682–689.
161 Peerschke EI. Time-dependent association between platelet-bound fibrinogen and the triton X-100 insoluble cytoskeleton. *Blood* 1991; 77: 508–514.
162 Wencel-Drake JD, Boudignon-Proudhon C, Dieter MG, Criss AB, Parise LV. Internalization of bound fibrinogen modulates platelet aggregation. *Blood* 1996; 87: 602–612.
163 Plow EF, D'Souza SE, Ginsberg MH. Consequences of the interaction of platelet membrane glycoprotein GPIIb–IIIa ($\alpha_{IIb}\beta_3$) and its ligands. *J Lab Clin Med* 1992; 120: 198–204.
164 Frelinger AL, III, Lam SCT, Plow EF, Smith MA, Loftus JC, Ginsberg MH. Occupancy of an adhesive glycoprotein receptor modulates expression of an antigenic site involved in cell adhesion. *J Biol Chem* 1988; 263: 12397–12402.
165 Frelinger AL, III, Cohen I, Plow EF et al. Selective inhibition of integrin function by antibodies specific for ligand-occupied receptor conformers. *J Biol Chem* 1990; 265: 6346–6352.
166 Kouns WC, Wall CD, White MM, Fox CF, Jennings LK. A conformation-dependent epitope of human platelet glycoprotein IIIa. *J Biol Chem* 1990; 265: 20594–20601.
167 Frelinger AL, III, Du X, Plow EF, Ginsberg MH. Monoclonal antibodies to ligand-occupied conformers of integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa) alter receptor affinity, specificity and function. *J Biol Chem* 1991; 266: 17106–17111.
168 Jennings LK, White MM. Expression of ligand-induced binding sites on glycoprotein IIb/IIIa complexes and the effect of various inhibitors. *Am Heart J* 1998; 135: S179–S183.
169 Jennings LK, Haga JH, Slack SM. Differential expression of a ligand induced binding site (LIBS) by GPIIb–IIIa ligand recognition peptides and parenteral antagonists. *Thromb Haemost* 2000; 84: 1095–1102.
170 Bednar B, Cook JJ, Holahan MA et al. Fibrinogen receptor antagonist-induced thrombocytopenia in chimpanzee and rhesus monkey associated with preexisting drug-dependent antibodies to platelet glycoprotein IIb IIIa. *Blood* 1999; 94: 587–599.
171 Zamarron C, Ginsberg MH, Plow EF. A receptor-induced binding site in fibrinogen elicited by its interaction with platelet membrane glycoprotein IIb–IIIa. *J Biol Chem* 1991; 266: 16193–16199.

Cambridge Books Online © Cambridge University Press, 2009

172 Abrams CS, Ellison N, Budzynski AZ, Shattil SJ. Direct detection of activated platelets and platelet-derived microparticles in humans. *Blood* 1990; 75: 128–138.
173 Ugarova TP, Budzynski AZ, Shattil SJ, Ruggeri ZM, Ginsberg MH, Plow EF. Conformational changes in fibrinogen elicited by its interaction with platelet membrane glycoprotein GPIIb–IIIa. *J Biol Chem* 1993; 268: 21080–21087.
174 Du X, Gu M, Weisel JW et al. Long range propagation of conformational changes in integrin $\alpha_{IIb}\beta_3$. *J Biol Chem* 1993; 268: 23087–23092.
175 Clark EA, Brugge JS. Integrins and signal transduction pathways: The road taken. *Science* 1995; 268: 233–239.
176 Shattil SJ, Ginsberg MH. Perspective Series: Cell adhesion in vascular biology. Integrin signalling in vascular biology. *J Clin Invest* 1997; 100: S91–S95.
177 George JN, Caen JP, Nurden AT. Glanzmann's thrombasthenia: the spectrum of clinical disease. *Blood* 1990; 75: 1383–1395.
178 Rooney MM, Parise LV, Lord ST. Dissecting clot retraction and platelet aggregation. *J Biol Chem* 1996; 271: 8553–8555.
179 Barbieri B, Balconi G, Dejana E, Donati MB. Evidence that vascular endothelial cells can induce the retraction of fibrin clots. *Proc Soc Exp Biol Med* 1981; 168: 204–207.
180 Yee KO, Rooney MM, Giachelli CM, Lord ST, Schwartz SM. Role of β_1 and β_3 integrins in human smooth muscle cell adhesion to and contraction of fibrin clots in vitro. *Circ Res* 1998; 83: 241–251.
181 Law DA, DeGuzman FR, Heiser P, Ministri-Madrid K, Killeen N, Phillips DR. Integrin cytoplasmic tyrosine motif is required for outside-in alphaIIbbeta3 signalling and platelet function. *Nature* 1999; 401: 808–811.
182 Hawiger J, Timmons S, Kloczewiak M, Strong DD, Doolittle RF. Gamma and alpha chains of human fibrinogen possess sites reactive with human platelet receptors. *Proc Natl Acad Sci USA* 1982; 79: 2068–2071.
183 Kloczewiak M, Timmons S, Hawiger J. Recognition site for the platelet receptor is present on the 15-residue carboxy-terminal fragment of the gamma chain of human fibrinogen and is not involved in the fibrin polymerization reaction. *Thromb Res* 1983; 29: 249–255.
184 Plow EF, Pierschbacher MD, Ruoslahti E, Marguerie GA, Ginsberg MH. The effect of Arg–Gly–Asp-containing peptides on fibrinogen and von Willebrand factor binding to platelets. *Proc Natl Acad Sci USA* 1985; 82: 8057–8061.
185 Gartner TK, Bennett JS. The tetrapeptide analogue of the cell attachment site of fibronectin inhibits platelet aggregation and fibrinogen binding to activated platelets. *J Biol Chem* 1985; 260: 11891–11894.
186 Plow EF, Marguerie GA, Ginsberg M. Fibrinogen, fibrinogen receptors and the peptides that inhibit these interactions. *Biochem Pharmacol* 1987; 36: 4035–4040.
187 Andrieux A, Hudry-Clergeon G, Ryckwaert J-J et al. Amino acid sequences in fibrinogen mediating its interaction with its platelet receptor, GPIIb–IIIa. *J Biol Chem* 1989; 264: 9258–9265.
188 Ginsberg M, Pierschbacher MD, Ruoslahti E, Marguerie GA, Plow EF. Inhibition of fibronectin binding to platelets by proteolytic fragments and synthetic peptides which support fibroblast adhesion. *J Biol Chem* 1985; 260: 3931–3936.
189 Beacham DA, Wise RJ, Turci SM, Handin RI. Selective inactivation of the arg–gly–asp-ser (RGDS) binding site in von Willebrand factor by site-directed mutagenesis. *J Biol Chem* 1992; 267: 3409–3415.
190 Ruoslahti E. RGD and other recognition sequences for integrins. *Annu Rev Cell Biol* 1996; 12: 697–715.
191 Ruoslahti E, Engvall E. Perspectives Series: cell adhesion in vascular biology. Integrins and vascular extracellular matrix assembly. *J Clin Invest* 1997; 99: 1149–1152.
192 Pierschbacher MD, Ruoslahti E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* 1984; 309: 30–33.
193 Lam SCT, Plow EF, Smith MA et al. Evidence that arginine-glycine-aspartic acid and fibrinogen gamma chain peptides share a common binding site on platelets. *J Biol Chem* 1987; 262: 947–950.
194 Plow EF, Marguerie GA. Inhibition of fibrinogen binding to human platelets by the tetrapeptide Glycyl-L-Prolyl-L-Arginyl-L-Proline. *Proc Natl Acad Sci USA* 1982; 79: 3711–3715.
195 Chen CS, Chou S-H, Thiagarajan P. Fibrin(ogen) peptide Bbeta 15–42 inhibits platelet aggregation and fibrinogen binding to activated platelets. *Biochemistry* 1988; 27: 6121–6126.
196 Farrell DH, Thiagarajan P, Chung DW, Davie EW. Role of fibrinogen alpha and gamma chain sites in platelet aggregation. *Proc Natl Acad Sci USA* 1992; 89: 10729–10732.
197 Farrell DH, Thiagarajan P. Binding of recombinant fibrinogen mutants to platelets. *J Biol Chem* 1994; 269: 226–231.
198 Holmbäck K, Danton MJS, Suh TT, Daugherty CC, Degen JL. Impaired platelet aggregation and sustained bleeding in mice lacking the fibrinogen motif bound by integrin $\alpha_{IIb}\beta_3$. *EMBO J* 1996; 15: 5760–5771.
199 Hu DD, Barbas CF, III, Smith JW. An allosteric Ca^{2+} binding site on the β_3-integrins that regulates the dissociation rate for RGD ligands. *J Biol Chem* 1996; 271: 21745–21751.
200 Egbertson MS, Chang CTC, Duggan ME et al. Non-peptide fibrinogen receptor antagonists. 2. Optimization of a tyrosine template as a mimic for Arg–Gly–Asp. *J Med Chem* 1994; 37: 2537–2551.
201 Askew BC, Bednar RA, Bednar B et al. Non-peptide glycoprotein IIb/IIIa inhibitors. 17. Design and synthesis of orally active, long-acting non-peptide fibrinogen receptor antagonists. *J Med Chem* 1997; 40: 1779–1788.
202 Alig L, Edenhofer P, Hadvary P et al. Low molecular weight, non-peptide fibrinogen receptor antagonists. *J Med Chem* 1992; 35: 4393–4407.
203 Pytela R, Pierschbacher MD, Ruoslahti E. Identification and isolation of a 140 kd cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell* 1985; 40: 191–198.
204 Argraves WS, Pytela R, Suzuki S, Millan JL, Pierschbacher MD,

Cambridge Books Online © Cambridge University Press, 2009

Ruoslahti E. cDNA sequences from the alpha subunit of the fibronectin receptor predict a transmembrane domain and a short cytoplasmic peptide. *J Biol Chem* 1986; 261: 12922–12924.

205 Piotrowicz RS, Orchekowski RP, Nugent DJ, Yamada KY, Kunicki TJ. Glycoprotein Ic-IIa functions as an activation-independent fibronectin receptor on human platelets. *J Cell Biol* 1988; 106: 1359–1364.

206 Ginsberg MH, Du X, O'Toole TE, Loftus JC, Plow EF. Platelet integrins. *Thromb Haemost* 1993; 70: 87–93.

207 Suehiro K, Gailit J, Plow EF. Fibrinogen is a ligand for integrin $\alpha_5\beta_1$ on endothelial cells. *J Biol Chem* 1997; 272: 5360–5366.

208 Pytela R, Pierschbacher MD, Ruoslahti E. A 125/115-kDA cell surface receptor specific for vitronectin interacts with the arginine-glycine-aspartic acid adhesion sequence derived from fibronectin. *Proc Natl Acad Sci USA* 1985; 83: 5766–5770.

209 Cheresh DA. Human endothelial cells synthesize and express an Arg–Gly–Asp-directed adhesion receptor involved in attachment to fibrinogen and von Willebrand factor. *Proc Natl Acad Sci USA* 1987; 84: 6471–6475.

210 Lam SC, Plow EF, D'Souza SE, Cheresh DA, Frelinger AL, III, Ginsberg MH. Isolation and characterization of a platelet membrane protein related to the vitronectin receptor. *J Biol Chem* 1989; 264: 3742–3749.

211 Lawler J, Hynes RO. An integrin receptor on normal and thrombasthenic platelets that bind thrombospondin. *Blood* 1989; 74: 2022–2027.

212 Cheresh DA, Berliner SA, Vicente V, Ruggeri ZM. Recognition of distinct adhesive sites on fibrinogen by related integrins on platelets and endothelial cells. *Cell* 1989; 58: 945–953.

213 Smith JW, Ruggeri ZM, Kunicki TJ, Cheresh DA. Interaction of integrins $\alpha_v\beta_3$ and glycoprotein IIb–IIIa with fibrinogen. *J Biol Chem* 1990; 265: 12267–12271.

214 Smith RA, Rooney MM, Lord ST, Mosesson MW, Gartner TK. Evidence for new endothelial cell binding sites on fibrinogen. *Thromb Haemost* 2000; 84: 819–825.

215 Yokoyama K, Zhang X-P, Medved L, Takada Y. Specific binding of integrin $\alpha_V\beta_3$ to the fibrinogen gamma and α_E chain C-terminal domains. *Biochemistry* 1999; 38: 5872–5877.

216 Smith JW, Piotrowicz RS, Mathis D. A mechanism for divalent cation regulation of β_3-integrins. *J Biol Chem* 1994; 269: 960–967.

217 Suehiro K, Smith JW, Plow EF. The ligand recognition specificity of β_3 integrins. *J Biol Chem* 1996; 271: 10365–10371.

218 Coller BS, Cheresh DA, Asch E, Seligsohn U. Platelet vitronectin receptor expression differentiates Iraqi-Jewish from Arab patients with Glanzmann thrombasthenia in Israel. *Blood* 1991; 77: 75–83.

219 Felding-Habermann B, Cheresh DA. Vitronectin and its receptors. *Curr Opin Cell Biol* 1993; 5: 864–868.

220 Brooks PC. Role of integrins in angiogenesis. *Eur J Cancer* 1996; 32: 2423–2429.

221 Varner JA, Cheresh DA. Integrins and cancer. *Curr Opin Cell Biol* 1996; 8: 724–730.

222 Dvorak HF, Senger DR, Dvorak AM. Fibrin as a component of the tumor stroma: origins and biological significance. *Cancer Metastasis Rev* 1983; 2: 41–73.

223 Gasic GJ, Gasic TB, Stewart CC. Antimetastatic effects associated with platelet reduction. *Proc Natl Acad Sci USA* 1968; 61: 46–52.

224 Bennett JS, Chan C, Vilaire G, Mousa SA, DeGrado WF. Agonist-activated $\alpha_V\beta_3$ on platelets and lymphocytes binds to the matrix protein osteopontin. *J Biol Chem* 1997; 272: 8137–8140.

225 Gartner TK, Williams DC, Phillips DF. Platelet plasma membrane lectin activity. *Biochemical and Biophysical Research Commun* 1977; 79: 592–599.

226 Gartner TK, Phillips DR, Williams DC. Expression of thrombin-enhanced platelet lectin activating is controlled by secretion. *FEBS Lett* 1980; 113: 196–200.

227 Aiken ML, Ginsberg MH, Plow EF. Divalent cation-dependent and independent surface expression of thrombospondin on thrombin-stimulated human platelets. *Blood* 1987; 69: 58–64.

228 Aiken ML, Ginsberg MH, Plow EF. Identification of a new class of inducible receptors on platelets: thrombospondin interacts with platelets via a GPIIb–IIIa mechanism. *J Clin Invest* 1986; 78: 1713–1716.

229 Lawler J, Weinstein R, Hynes RO. Cell attachment to thrombospondin: The role of arg-gly-asp, calcium and integrin receptors. *J Cell Biol* 1988; 107: 2351–2361.

230 Asch AS, Barnwell J, Silverstein RL, Nachman RL. Isolation of the thrombospondin membrane receptor. *J Clin Invest* 1987; 79: 1054–1061.

231 Catimel B, Leung L, El Ghissasi H, Mercier N, McGregor J. Human platelet glycoprotein IIIb binds to thrombospondin fragments bearing the *C*-terminal region, and/or the type I repeats (CSVTCG motif), but not to the *N*-terminal heparin-binding region. *Biochem J* 1992; 284: 231–236.

232 Leung LLK, Li WX, McGregor JL, Albrecht G, Howard RJ. CD36 peptides enhance or inhibit CD36-thrombospondin binding – a 2-step process of ligand-receptor interaction. *J Biol Chem* 1992; 267: 18244–18250.

233 San Antonio JD, Slover J, Lawler J, Karnovsky MJ, Lander AD. Specificity in the interactions of extracellular matrix proteins with subpopulations of the glycosaminoglycan heparin. *Biochem J* 1993; 32: 4746–4755.

234 Leung LLK, Nachman RL. Complex formation of platelet thrombospondin with fibrinogen. *J Clin Invest* 1982; 70: 542–549.

235 Bacon-Baguley T, Kudryk BJ, Waltz DA. Thrombospondin interaction with fibrinogen. *J Biol Chem* 1987; 262: 1927–1930.

236 Bonnefoy A, Hantgan R, Legrand, C, Frojmovic MM. A model of platelet aggregation involving multiple interactions of thrombospondin-1, fibrinogen, and GPIIbIIIa receptor. *J Biol Chem* 2001; 276: 5605–5612.

237 Hantgan RR. Fibrin protofibril and fibrinogen binding to ADP-stimulated platelets: Evidence for a common mechanism. *Biochim Biophys Acta* 1988; 968: 24–35.

238 Hantgan RR, Ramsamooj P, Taylor RG, Lewis JC. Why does

Cambridge Books Online © Cambridge University Press, 2009

fibrin bind so tightly to stimulated platelets? In MW Mosesson et al, eds. *Fibrinogen 3. Biochemistry, biological functions, gene regulation and expression*. Elsevier Science Publishers BV 1989; 203–206.

239 Lewis JC, Johnson C, Ramsamooj P, Hantgan RR. Orientation and specificity of fibrin protofibril binding to ADP-stimulated platelets. *Blood* 1988; 72: 1992–2000.

240 Ward CM, Kestin AS, Newman PJ. A Leu262Pro mutation in the integrin β_3 subunit results in an α_{IIb}-β_3 complex that binds fibrin but not fibrinogen. *Blood* 2000; 96: 161–169.

241 Hantgan RR, Hindriks G, Taylor RG, Sixma JJ, de Groot PG. Glycoprotein Ib, von Willebrand factor, and glycoprotein IIb–IIIa are all involved in platelet adhesion to fibrin in flowing whole blood. *Blood* 1990; 76: 345–353.

242 Niewiarowski S, Levy-Toledano S, Caen JP. Platelet interaction with polymerizing fibrin in Glanzmann's thrombasthenia. *Thromb Res* 1981; 23: 457–463.

Cambridge Books Online © Cambridge University Press, 2009

14

Platelet signalling: GTP-binding proteins

Jan-Willem N. Akkerman

Laboratory for Thrombosis and Haemostasis, Department of Haematology, University Medical Center Utrecht, The Netherlands

Introduction

To respond to extracellular signals, platelets possess different types of surface receptors for stimulatory and inhibitory ligands. Surface receptors can be classified in three groups each possessing a specific property by which information is relayed through the plasma membrane. These are: (i) G-protein linked receptors, (ii) enzyme linked receptors and, (iii) ion channel-linked receptors. G-protein linked receptors are seven transmembrane receptors that relay the stimulus to high molecular weight, heterotrimeric GTP-binding regulatory proteins (G-proteins in short) consisting of three polypeptides, an α-, a β- and a γ-subunit. Enzyme linked receptors have a single transmembrane domain and contain a cytosolic tail with an intrinsic enzyme activity or that associates directly with an enzyme. They start signalling cascades that involve a second class of GTP-binding proteins which are low molecular weight, monomeric GTPases (small GTPases in short). Ion channel linked receptors respond directly to ligand binding by opening a channel pore resulting in single channel currents.

Both G-proteins and small GTPases are members of the superfamily of GTPases. They function as molecular switches between a GTP-bound 'on-state' and a GDP-bound 'off-state'. Hence, they are important elements in the initiation and termination of signal transduction. In addition, they serve in amplification of signals helping to convert a weak extracellular stimulus into a strong intracellular signal. The fact that one G-protein might be connected to several receptors or couple to more than one downstream effector enables integration of different signals. Thus, GTP-binding proteins are key elements in the signalling cascades that initiate shape change, aggregation, secretion and contraction, or prevent this [1–5].

G-proteins and small GTPases

G-proteins

The classification 'G-protein linked receptor' is based on the property that ligand binding to these receptors starts the activation of G-proteins. These receptors are typically heptahelical receptors, consisting of seven stretches of about 22 hydrophobic residues. These regions are thought to form transmembrane α-helices with a loop between helices 5 and 6 facing the cytosol which together with the C-terminal end forms the binding site for the G-protein. G-proteins consist of an α-, a β- and a γ subunit with mol.weights 39–52 kDa, 35–36 kDa and 5–8 kDa, respectively. The $\beta\gamma$ subunit splits only in the presence of detergents and under physiological conditions forms a functional monomer[1]. The α subunit contains a GTPase domain with the GTP-binding pocket as well as sites for binding of receptors, effectors and $\beta\gamma$ complexes. The C-terminus and parts of the α5 helix are sites for interaction with the receptor and thus determine receptor specificity[1]. In the absence of ligands both GDP-α and $\beta\gamma$ are bound to the receptor, α binding being greatly facilitated by $\beta\gamma$ (Fig. 14.1(*a*)). Ligand binding induces a conformational change in the receptor that moves the C-terminal α-helix of the α-subunit. This causes dissociation of bound GDP which is easily replaced by GTP as this is present in excess. The GTP-α-subunit is activated and dissociates from the receptor and from the $\beta\gamma$-complex. Each subunit can then activate their target effector. The intrinsic GTPase activity within the α-subunit restores the GDP-bound state, enabling the α-subunit to reassociate with the $\beta\gamma$-complex and the cycle is closed. GTP hydrolysis is under control of regulators of G-protein signalling. The system has an important feed back mechanism since the release of active G-protein triggers a decrease in ligand affinity of the receptor. Thus, initi-

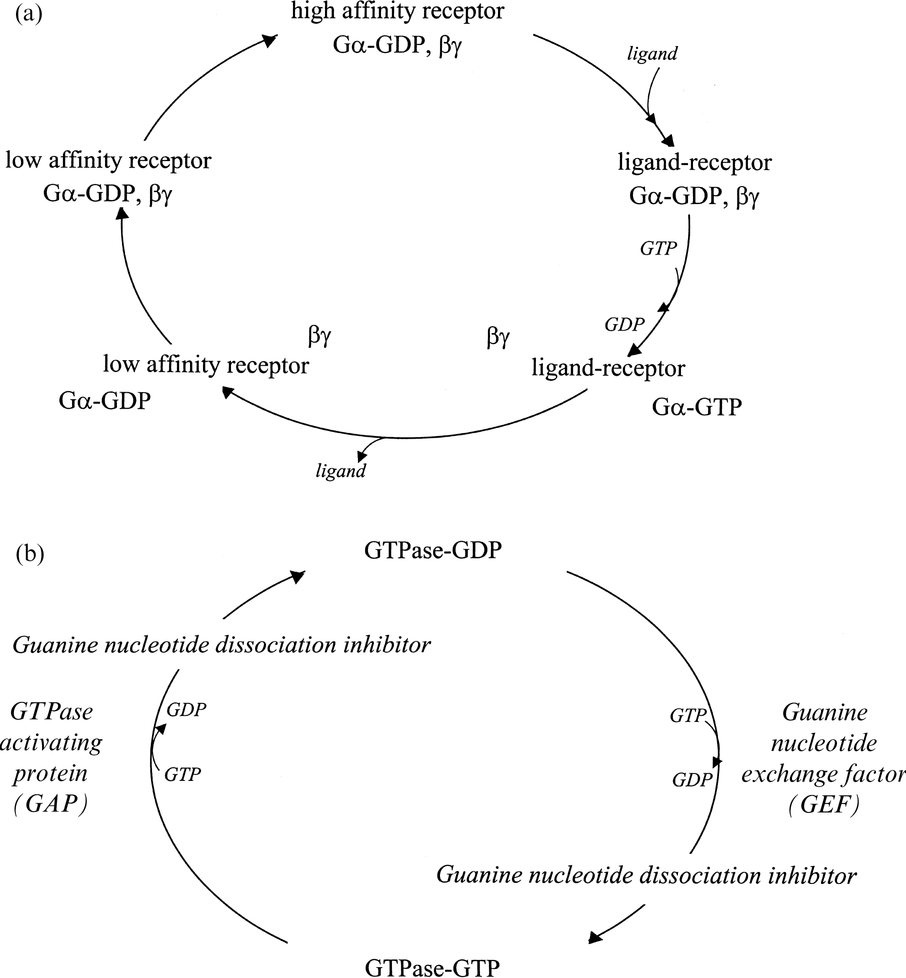


Fig. 14.1. Activation and deactivation of GTP-binding proteins. (*a*) G-proteins; (*b*) small GTPases.

ation of downstream signalling by active G-protein is accompanied by termination of receptor activation unless the ligand concentration is sufficiently high to start a new activation signal[1,4].

GTP is bound in a cleft with the purine located near the cleft entrance and the phosphate buried within the protein[6]. This core, also called 'G-domain', is almost identical for all G-proteins. The activation of Gα-subunits by GTP implies that the protein senses a relatively small change in the active site: the presence or absence of a γ-phosphate. This sensor resides upon the key residues Thr and Gly in a special region in the G-domain (G-2 region also called switch 1). GTP binding results in movement of the G-2 and adjacent G-3 (switch II) domains, which activates the Gα subunit[6]. Popular tools to activate G-proteins are AlF_4^-, which mimicks the GTP-induced movement of the G-2 and G-3 regions, and GTPγS or GMP-P(NH)P, which are resistant to hydrolysis. On the other hand, GDPβS interferes with GTP binding, inducing persistent inactivation.

More than 20 different α-subunits have been identified. Diversity is lower for β-subunits (about 5) and γ-subunits (about 10) and although in theory this would lead to numerous combinations, only a limited number of $\beta\gamma$-complexes exist. Specificity in β for binding to a γ-subunit lies in the so called WD domains. Conversely, a stretch of 14 amino acids in the middle of γ determines specificity for β-binding.

G-proteins are divided according to amino acid similarity in four classes: the α_s-, α_i-, α_q- and α_{12}-classes. The α-subunits can be phosphorylated on Ser, Thr or Tyr residues, the latter by pp60 Src, which changes the interaction with other regulatory proteins.

Cambridge Books Online © Cambridge University Press, 2009

G_s contains the α_s-subunit and stimulates adenylyl cyclase leading to the formation of cAMP. Two products of alternative splicing are known, a short α_s (45 kDa) and a long α_s (52 kDa). A tool to investigate the role of G_s is cholera toxin, which catalyses NAD-dependent ADP-ribosylation of Arg 187/188 (Arg 201/202 in splice variants) of $G_s\alpha$ subunit inducing persistent activation due to impaired GTP-hydrolysis.

G_i contains the α_i-subunit (40 kDa) and is the physiological counterpart of G_s as it inhibits adenylyl cyclase thereby abolishing cAMP formation. There are at least three isoforms (G_i α_1, α_2, α_3), with additional diversity in downstream signalling. Pertussis toxin catalyses NAD-dependent ADP-ribosylation of a Cys residue close to the C-terminus of $G_i\alpha$ (and of $G_o\alpha$ and $G_t\alpha$ which are absent in platelets), which blocks receptor binding to the α-subunit and inhibits signalling through this G-protein. Also NEM is an inhibitor of G_i proteins. On the other hand, G_i is activated by mastoparan and mastoparan analogue Mas7[7]. A special member of this class is G_z (41 kDa). Expression studies show interaction with Rap1GAP, the GTPase that inactivates the small GTPase Rap1[8].

G_q is in fact a class of different, pertussis toxin-insensitive G-proteins consisting of α_q (42 kDa), which activates phospholipase $C\beta_2$, and α_{15} (in mice platelets)/α_{16} (in human platelets; 43.5 kDa[9]), which appears specific for haematopoietic cells. $G_{16}\alpha$ antisense expressed in HEL cells leaves signalling sequences induced by thrombin and platelet activating factor intact, but changes growth factor signalling[10].

G_{12} is also a class of multiple G-proteins such as those consisting of α_{12} (44 kDa) and α_{13} (43 kDa[11]). These G-proteins signal to a special class of small GTPases that control actin polymerization.

Binding to membranes is an important prerequisite for G-protein function. The α_i-subunits are cotranslationally modified by myristoylation at N-terminal glycine, which is an irreversible process that facilitates $\beta\gamma$ binding. In contrast, α_s and α_q are not myristoylated. The α_s is post-translationally modified by palmitoylation at Cys 3 which is a reversible process enabling an α subunit to switch between a palmitoylated, active state and a depalmitoylated, inactive state. The $\beta\gamma$ subunits are independent activators of multiple signalling pathways. The prenylgroup at the C-terminus in γ mediates the binding of $\beta\gamma$ to the membrane. The binding to GDP-α blocks the signalling properties of $\beta\gamma$. The $\beta\gamma$ complexes bind to α-subunits, receptors, adenylyl cyclases, phospholipase C_β, calmodulin, cytosolic phospholipase A_2, phosphatidylinositol 3-kinase (PtdIns 3-kinase), among others.

Small GTPases

Small GTPases take part in the signalling cascades initiated by enzyme linked receptors. In addition, they serve in many other aspects of cell function, e.g. vesicle transport and cytoskeleton assembly. These monomeric, 20–30 kDa molecular weight proteins, have a similar GTP–GDP dependent on/off system to the trimeric G-proteins, but the activation cycle is under strict control of proteins that facilitate GTP binding or enhance its hydrolysis (figure 14.1(*b*)). GTP binding is catalysed by guanine nucleotide exchange factors or GEFs, most of them being specific for a single type of small GTPase. GTP-binding induces a conformational change in the so-called switch 1 and switch 2 regions, the former mediating the interaction with downstream effectors. Small GTPases contain an intrinsic GTPase activity but its velocity is low. Hence, GTP hydrolysis is catalysed by GTPase activating proteins or GAPs. Further control of the GTPase cycle comes from guanine nucleotide dissociation inhibitors or GDIs, which inhibit the release of GDP or GTP from the GTPase. In addition, adapter proteins that lack enzymatic activity assist in docking of exchange factors and GTPases. All small GTPases are posttranslationally modified by limited hydrolysis at the C-terminus, followed by isoprenylation and carboxymethylation. The prenyl moiety is farnecyl for GTPases ending with Ser, Met or Gln, or geranylgeranyl for those ending with Leu. This reaction is essential for membrane binding and proper function.

Those small GTPases that transduce signals from enzyme linked receptors are anchored to the activated receptors following ligand induced receptor dimerization, which triggers autophosphorylation of specific domains, mostly Tyr residues. These become the docking sites for adapter proteins with Src-homology (SH) domains by which they couple to the receptor dimer (SH2 domains) and to a specific GEF (SH3 domains). The result is GDP-GTP exchange of the small GTPase and activation. In a next step, downstream signalling is initiated by activating a target kinase or by docking to the exchange factor of a next effector.

The small GTPases are subdivided in the families of the Ras-like proteins, Rho-like proteins, Rab proteins, Arf proteins, Ran proteins and Rad proteins. A simplified functional classification is that Ras members function in the regulation of cell growth, Rho members in control of the actin cytoskeleton, the Rab- and Arf-members in vesicle fusion and transport and the Ran members in transport across the nuclear membrane. The function of Rad proteins is still elusive.

The Ras subfamily (21–30 kDa) consists of 17 proteins

Cambridge Books Online © Cambridge University Press, 2009

including H-Ras, R-Ras, K-Ras, Rap, and Ral among others. Ras-like GTPases are post-translationally modified by prenylation by a farnecyl protein transferase, which couples a farnecylisoprenoid moiety to a Cys residue in a C-terminal CAAX motif. This enables coupling to the membrane which is essential for Ras function[12]. RasGEFs include Sos, which couples receptor tyrosine kinases to Ras and is an exchange factor. GAPs for Ras are p120GAP and NF1. A major effector of Ras-GTP is the Ser/Thr kinase Raf, which in turn activates the ERK1/2 kinases (also called p42/44 MAPkinases). A second effector of Ras is PtdIns 3-kinase, which generates PtdIns 3,4,5 P_3. This is a first step in downstream signalling to protein kinase B[13], which is an anti-apoptotic kinase, and to protein kinase C, a key factor in platelet aggregation and secretion. Different Ras members differ in their capacity to initiate the different downstream pathways. Many other Ras effectors have now been described including Rlf, which is a GEF for Ral, protein kinase Cζ and JNK, a member of the MAPkinase family.

Rap1 (21 kDa) and Ras share about 50% homology, but their effector domains are virtually identical[14,15]. Rap1 is present as Rap1A and Rap1B, differing only in 9 out of 184 aminoacids. Both are postranslationally modified at the C-terminus by a geranylgeranyl moiety that mediates membrane attachment. Exchange factors for Rap1 include CalDAG-GEF, regulated by Ca^{2+}/diacylglycerol and Epac, regulated by cAMP[16]. SPA-1 is a Rap-GAP. Interestingly, transient overexpression of SPA-1 in cell lines inhibits Rap1 activation as well as adhesion to a fibronectin-coated surface suggesting that Rap might function in signalling through integrins[17].

Rho-like GTPases (20–30 kDa) are about 30% homologous to Ras[18,19]. Several subtypes have been identified, e.g. Rho A/B/C, Rac 1/2/3, Cdc42 and G25K[19]. The Rho members are involved in the formation of stress fibres and the assembly of focal adhesions. Rac members function in the formation of lamellipodia, membrane ruffles and focal contacts and Cdc42 is important for formation of filopodia and focal contacts.

GTP–GDP exchange is regulated by RhoGEFs that are characterized by a common Dbl-m homology (DH) domain. This encodes the catalytic activity and a pleckstrin homology (PH) domain, which mediates membrane attachment[18]. About 20 RhoGAPs have been identified. Rho-like GTPases bind to membranes via geranylgeranyl lipid modification, but in the inactive state Rho's are found in the cytosol bound to Rho GDIs. The exoenzyme C3 transferase, which is an ADP-ribosyltransferase from *Clostridium botulinum* inactivates Rho A, Rho B and Rho C. A more general inhibitor of Rho-members is *C. difficile*. Effector activation by Rho-like GTPases generally involves disruption of an intramolecular autoinhibitory bond that exposes functional domains within the effector molecule. Examples are Rho-kinase, an effector of Rho, and Wiskott–Aldrich syndrome protein, an effector of Cdc42. Inhibition of Rho-kinase by the pharmacological inhibitor Y-27632 blocks formation of stress fibres, illustrating the role of Rho proteins in the regulation of actomyosin assembly and contraction.

An important effector of Rac-GTP is PtdIns 4–P5-kinase, which triggers the release of capping proteins and actin nucleation and polymerization. A second effector is WAVE, which also contributes to these processes[18].

Major effectors of Cdc42 are Wiskott Aldrich syndrome protein and the SerThr kinase PAK. WASP is expressed in haematopoietic cells and has a more ubiquitously expressed homologue called N-WASP. N-WASP, together with PAK, controls actin nucleation and filament stabilization, respectively.

An interesting group of effectors of Rac/Cdc42 are Mlks which act as MAPkinase kinase kinase (MEKKs). These enzymes activate the MAPkinase family members JNK and p38MAPkinase[18].

Rab GTPases function in the regulation of vesicular traffic. Upon GDP–GTP exchange, these proteins facilitate the binding of a transport vesicle to its proper acceptor organelle and initiate vesicle fusion. Arf proteins are activated by Golgi-bound enzymes and start the formation of transport vesicles.

G-proteins in platelets and megakaryocytes

G-proteins as integrators of signal transduction

Table 14.1 lists the major G-proteins in human platelets. The diversity in signalling properties mainly reflects the differences in the α subunit. However, also $\beta\gamma$ complexes can signal to downstream enzymes or ion channels but it is often uncertain from which trimeric complexes they originate. There are clear examples of G-proteins that are activated by different receptors, illustrating their role in integration of different extracellular signals. There appears to be less diversity in downstream effectors but as more information on isozyme distribution becomes available, a further complexity in downstream signalling will become apparent[20–24].

The stimulatory G-protein of adenylyl cyclase Gs

A major inhibitor of platelet functions is cAMP and its control by activating and inhibitory signalling cascades is a

Cambridge Books Online © Cambridge University Press, 2009

Table 14.1. G-proteins in platelets: coupling with receptors

Protein	Surface receptor	Activator	Refs	Effector	Refs
$G_s\ \alpha$	IP-receptor	PGI_2	134,135	adenylylcyclase (+)	23,136
	EP_2-receptor	PGE_2	136	MHC-1	27
	DP-receptor	PGD_2	23		
	A_{2A} adenosine receptor	adenosine	23		
	β_2-adrenergic receptor	epinephrine	23		
$G_i\ \alpha$	PAR1/3/4	thrombin	35,137	adenylylcyclase (−)	137
	$P2T_{AC}$ ($P2Y_{12}$)	ADP	32,37,40,41,53	integrin ass. protein	44
	α_{2A}-adrenergic receptor	epinephrine	41,53	Na+/H+ exchanger (−)	7,45
	paf-1-receptor	paf-acether	23	SH-PTP-1	46
	Edg 2/4/7 receptor	lysophosphatidic acid	34,62		
	Edg 1/3/5 receptor	sphingosine-1 phosphate	34,62		
$G_q\ \alpha$	PAR1/3/4	thrombin	11,50,137,138	phospholipase Cβ2 (+)	50
	$P2Y_1$	ADP	40,41,50,53		
	TPα/β receptor	thromboxane A_2	11,32,50,53,60,133		
	$5HT_{2A}$ receptor	serotonin	41,53		
	paf-1-receptor	paf-acether			
	V-1 vasopressin receptor	arginine-vasopressin	139		
$G_{12}\ \alpha$	PAR1/3/4	thrombin	11,59	Rho kinase (+)	54
	$P2T_{AC}$ ($P2Y_{12}$)	ADP	32	pp60 src, pp72 syk	
	TPα/β receptor	thromboxane A_2	11,59		
$G_{13}\ \alpha$	PAR1/3/4	thrombin	11	Rho kinase (+)	34,54,62
	TPα/β receptor	thromboxane A_2	11,54,59,60,61,117,140	pp60 src, pp72 syk	
	Edg 2/4/7 receptor	lysophosphatidic acid			
$G_{16}\ \alpha$	TPα/β receptor (?)	thromboxane A_2	9,63	phospholipase Cβ2 (+)	
$G_z\ \alpha$	α_{2A}-adrenergic receptor	epinephrine	64,141	adenylyl cyclase (+)	64
$G_h\ \alpha$	TPα-receptor	thromboxane A_2	67	phospholipase Cβ2 (+)	67
G $\beta\gamma$	derived from G_i, G_q	G_i/G_q coupled receptors	70,72	phospholipase Cβ2 (+)	70
				phospholipase Cβ3 (+)	
				PtdIns 3-kinase γ (+)	

Notes:
$G_{11}\alpha$ is absent in platelets[9,25,132,133].
Platelets contain TPβ mRNA but little protein[55].
Abbreviations used: (+) and (−) indicate activation and inhibition, respectively.

nice illustration of the action of G-proteins (Fig. 14.2). The G-protein G_s (for stimulatory) is activated by binding of PGI_2 (prostacyclin) and a number of other inhibitory prostaglandins to their respective receptors. Adenosine activates the A_{2A} adenosine receptor, which also activates G_s. Under normal conditions epinephrine activates platelets via the α_{2A}-adrenergic receptor but when this receptor is blocked a second property becomes apparent which is the result of binding to β_2-adrenergic receptors and activation of G_s. The result of G_s activation is activation of adenylyl cyclase, accumulation of cAMP and inhibition of numerous signalling steps involved in platelet activation mainly via protein kinase A.

Analysis by RT-PCR of platelet mRNA reveals a short and a long form (called $G_s\alpha$-S and $G_s\alpha$-L, respectively). The two forms differ in 45 base pairs and are products of alternative splicing. In addition, each form is present without and with a C-A-G triplet. The triplet results in a consensus sequence (Ser–X-Lys) for a phosphorylation site for the Ser/Thr kinase protein kinase C, making these variants targets for control of cAMP formation by this kinase[25]. In accordance with its role in supporting surface receptors, $G_s\alpha$ is pre-

Cambridge Books Online © Cambridge University Press, 2009

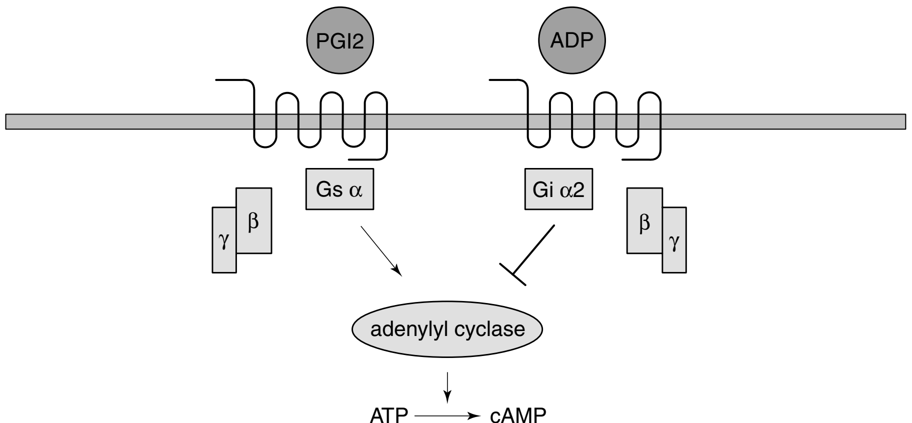


Fig. 14.2. Regulation of adenylyl cyclase by G-proteins. Prostacylcin (PGI_2) and ADP activate the IP-receptor, and the $P2T_{AC}$-receptor respectively. In turn, this activates G_s and G_i, the stimulatory and inhibitory G-protein for adenylyl cyclase. The result is production of cAMP or its inhibition.

dominant in plasma membranes, but also found in dense tubular membranes and in dense granules. Possibly it is part of a SNAP receptor complex which is known to contain G-protein subunits and assists in vesicle targeting and fusion[26]. In PGI_2-treated platelets $G_s\alpha$ physically associates with the Major Histocompatability Complex, MHC-1[27].

$G_s\alpha$ mRNA and protein are already expressed in the megakaryoblastic cell line MEG-01, which represents an early stage in megakaryocytopoiesis[28]. Stimulation with cholera toxin or the PGI_2 analog iloprost raises cAMP in MEG-01 by 2–4 fold. Co-stimulation with thrombin upregulates the PGI_2-induced cAMP formation, a property that is abolished by protein kinase C inhibition. This is quite different from cAMP control in platelets, where thrombin suppresses cAMP formation via an inhibitory G-protein as well as via protein kinase C.

The megakaryoblastic cell lines MEG-01, DAMI and CHRF 288–11 have properties in common with normal megakaryocytes at different stages of maturation. Total $G_s\alpha$ mRNA and protein are down-regulated at increasing stages of maturation, which is accompanied by a decrease in the ratio $G_s\alpha$-S / $G_s\alpha$-L and up-regulation of cAMP formation[28,29]. This accords with the kinetic properties of $G_s\alpha$-L, which is 3–10 fold more active than the short form. Platelets show the lowest $G_s\alpha$-S/ $G_s\alpha$-L ratio (0.8), which might explain their extreme sensitivity to PGI_2.

Platelets from patients with certain psychiatric disorders show increased G_s expression[30] or abnormal G_s activation[31].

The inhibitory G-protein of adenylyl cyclase G_i

The activation of adenylyl cyclase by G_s is counterbalanced by the inhibitory G-protein G_i. Thrombin, ADP, epinephrine and platelet activating factor stimulate platelets and at the same time lower cAMP by activating G_i[32,33]. Also lysophosphatidic acid and sphingosine-1 phosphate activate receptors coupled to G_i[34]. This mechanism guarantees that activating pathways function optimally and trigger full platelet responses. In the presence of inhibitory prostaglandins adenylyl cyclase-stimulating signals must be fully neutralized by inhibitory signals generated via G_i. Inhibition by G_i is not always sufficiently strong to prevent cAMP formation[35]. Thrombin activates platelets by proteolytic cleavage of three receptor subtypes: PAR1 (human platelets), PAR3 (mice platelets) and PAR4 (human and mice platelets[36]). The PAR1 activating peptide SFLLRN induces ADP-ribosylation of $G_i\alpha$, demonstrating coupling between PAR1 and G_i. However, inhibition of PGI_2-induced cAMP formation is incomplete and full suppression requires activation of all PAR subtypes.

Mice with a deleted gene for $G_i\alpha_2$ show strongly reduced ADP aggregation with a normal shape change[37]. Also the inside out activation of integrin $\alpha_{IIb}\beta_3$ following stimulation with ADP is severely impaired. $G_i\alpha_3$ partly compensates for the loss of $G_i\alpha_2$.

ADP stimulates platelets by activating the $P2Y_1$-receptor, which mediates platelet activating signals, the $P2T_{AC}$, which couples to adenylyl cyclase, and the P2X-1, which opens a calcium channel[38]. The $P2T_{AC}$ has been recently renamed $P2Y_{12}$[39]. Studies with ligand mimetics and receptor antagonists show that both the $P2Y_1$- and $P2T_{AC}$ receptors signal to optimal platelet aggregation (Fig. 14.3). Inhibition of the $P2T_{AC}$ inhibits ADP aggregation but has little effect on shape change, suggesting that this response is independent of G_i[40]. Activated α_{2A}-adrenergic receptors can replace $P2T_{AC}$ activation in assisting a $P2Y_1$ ligand to

Cambridge Books Online © Cambridge University Press, 2009

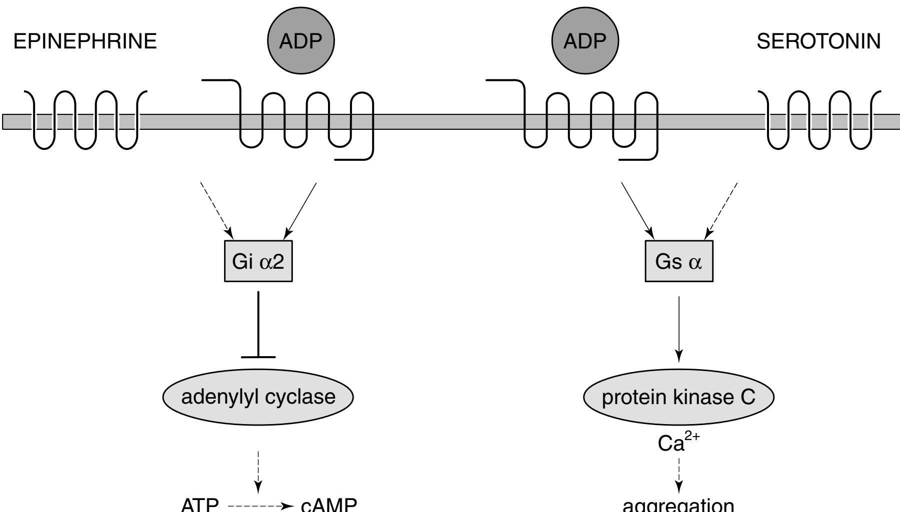


Fig. 14.3. Optimal aggregation depends on activation of both G_q and G_i. ADP induces aggregation by activating Gq via the $P2Y_1$-receptor. The result is a Ca^{2+} increase and protein kinase C activation resulting in aggregation and secretion. ADP also activates G_i (via the $P2T_{AC}$-receptor) thereby lowering cAMP and enabling optimal responses to occur. G_q activation by ligand – $P2Y_1$ complex can be replaced by ligand – $5HT_{2A}$ receptor complex; G_i activation by ligand – $P2T_{AC}$ complex can be replaced by ligand – α_{2A} adrenergic receptor complex.

fully activate the platelets. Alternatively, activation of the serotonin receptor supplements signalling through the $P2Y_1$[41]. This illustrates that activated G_i is the crucial element that lowers cAMP and enables optimal ADP-induced platelet aggregation to occur.

G_i may also take part in platelet activation by epinephrine. Activation of the α_{2A}-adrenergic receptor activates protein kinase C in a pertussis toxin sensitive manner[42]. In epinephrine-stimulated platelets pp60Src associates with $G_i\ \alpha$[43].

Inactive G_i has been found in a complex with Integrin Associated Protein (CD47), a thrombospondin receptor that forms a signalling complex with $\beta 3$ integrins. Aggregation induced via this receptor was abolished by pertussis toxin whereas GTP and AlF_4^- released G_i from the complex. This was accompanied by lowering of cAMP and reduced ligand binding to CD47[44].

Another effector of $G_i\alpha$-GTP is the Na^+/H^+ exchanger. This electron neutral pump removes protons from the cytosol in a stoichiometric exchange for extracellular Na^+ ions, thereby regulating the cytosolic pH (pH_i) and cell volume. In thrombin stimulated platelets receptor-mediated changes in pH_i are blocked following pretreatment with AlF_4^- and this inhibition is releaved by NEM. The exchanger preserves responsiveness to changes in the cytosolic proton concentration. These properties suggest an inhibitory role for G_i in receptor-mediated regulation of the Na^+/H^+ exchanger[7,45]. Another effector of G_i is SH-PTP1, a protein tyrosine phosphatase that contains two SH2 domains. Thrombin and PMA trigger phosphorylation on Ser and Tyr, thereby stimulating phosphatase activity. This coupling depends on G_i activation[46].

Platelets contain the G_i subtypes $G_i\ \alpha_2$ and $G_i\ \alpha_3$, which are 85–95% homologous at the aminoacid level[3]. $G_i\ \alpha_2$ and $G_i\ \alpha_3$ are present in the plasma membrane, dense tubular membranes and in dense granules. $G_i\ \alpha_2$ and $G_i\ \alpha_3$ are already expressed in MEG-01 and present in DAMI and CHRF 288–11. Treatment with the mastoparan analogue Mas 7 abolishes iloprost induced cAMP formation, indicating that G_i can be activated and suppresses adenylyl cyclase in these immature cells.

Decreased expression of platelet $G_i\alpha_2$ has been reported in major depression[47,48] and hypertension[49].

G_q, a key step in platelet activation

The crucial role of G_q in platelet activation is illustrated by the absent aggregation and secretion in G_q-deficient mouse platelets following stimulation by thrombin, ADP and thromboxane A_2[50]. The absent aggregation in G_q deficient platelets illustrates that activation of both G_i and G_q is required for a normal aggregation response[40,41,51–53]. Interestingly, G_q knock-out platelets show a normal shape change response following stimulation with thrombin or thromboxane A_2[54] and ADP[33]. A similar bypass of G-protein mediated signalling was found for G_i, suggesting that shape change is under control of different activation routes[54]. ADP-induced aggregation requires the concomi-

Cambridge Books Online © Cambridge University Press, 2009

tant stimulation of both G_q (via $P2Y_1$) and G_i (via $P2T_{AC}$). As described earlier, the response is preserved when the ligand for $P2Y_1$ is replaced by serotonin, illustrating that the serotonin receptor (5-HT_{2A} receptor) is also coupled to G_q. Transfection studies show that both splice variants of the thromboxane A_2 receptor, TPα and TPβ, couple to G_q[55].

G_q α is already expressed in MEG-01 and more mature megakaryoblastic cell lines. These cells respond to thrombin and platelet activating factor with a Ca^{2+} response, indicating that corresponding receptors and phospholipase $C\beta_2$ are present and functional[28].

Reduced expression of platelet G_q caused loss of responsiveness to thromboxane A_2 and an increased tendency to bleed[56]. Impaired G_q activity resulting in reduced responsiveness to thromboxane A_2 has been reported for neonatal platelets[57] and platelets from dogs[58].

G_{12} and G_{13}

The G-protein G_{12} is activated in platelets stimulated by thrombin, ADP and thromboxane A_2, indicating that this G-protein is coupled to the PAR receptors, the $P2Y_1$ receptor, and the TPα/β receptor, respectively[11,32,59]. Concurrently, $G\alpha_{12}$ is phosphorylated by protein kinase C, probably by the β,δ or ε subtype.

G_{13} is a member of the G_{12} class. $G_{13}\alpha$ is activated by thrombin, thromboxane A_2 and lysophosphatidic acid but not by ADP[32,60]. Evidence for coupling of $G_{13}\alpha$ to the TP receptor is based on copurification with the receptor, the fact that ligand binding to the receptor induces GTPγS incorporation in $G_{13}\alpha$ and the observation that the affinity of $G_{13}\alpha$ for receptor binding is changed upon ligand binding to the receptor[60]. $G_{13}\alpha$ is the target of dual phosphorylation. There is a protein kinase A mediated phosphorylation induced by prostacyclin, which results in arrest of thromboxane A_2-induced signalling[61]. A second phosphorylation is induced by protein kinase C in platelets activated by thrombin or thromboxane A_2[59].

Since stimulation with thromboxane A_2 activates G_q, G_{12} and G_{13} simultaneously, it is not easy to clarify the role of the individual G-proteins in platelet functions. Mouse platelets deficient in G_q are therefore of special interest. These cells lack thromboxane A_2-induced aggregation and secretion, but preserve a normal shape change response[50]. G_q knock-out platelets show a normal tyrosine phosphorylation of pp72 syk and pp60 c-src, suggesting that the tyrosine phosphorylation of these proteins is initiated by $G_{12/13}$. The normal shape change response is accompanied by phosphorylation of myosin-light chain, which is an important step in cytoskeleton assembly. Both shape change and myosin-light phosphorylation are abolished by inhibition of Rho-kinase, an effector of the small GTPase Rho. This suggests that $G_{12/13}$ is an upstream regulator of the Rho kinase – myosin light chain kinase pathway[54], possibly by activating the RhoGEFs p115RhoGEF and PDZRhoGEF. Lysophosphatidic acid binds to the Edg2/4/7 receptor which is coupled to G_{13} inducing Rho/Rho-kinase dependent shape change[34,62].

G_{15} / G_{16}

G_{15} (in mice platelets) / G_{16} (in human platelets) is a member of the Gq class of G-proteins and mainly expressed in haematopietic cells[9]. It is present in a short (43 kDa) and long (46 kDa) form[63]. $G_{16}\alpha$ lacks a cysteine that is the target for pertussis toxin ADP ribosylation. $G_{16}\alpha$ mRNA and protein are absent in MEG-01 cells but DAMI and CHRF-288–11 show increasing levels of expression of this subunit[28]. The appearance of $G_{16}\alpha$ mRNA and protein expression is accompanied by appearance of thromboxane A_2 induced signalling to Ca^{2+} mobilization and cAMP suppression. This raises the possibility that this G-protein couples the TP-receptor to phospholipase $C\beta_2$.

G_z

G_z is a member of the G_i family but resistant to pertussis toxin. The protein is highly expressed in platelets. $G_z\alpha$-deficient mice platelets show impaired reduction of cAMP by epinephrine, indicating that this G-protein functions in the coupling between the α_{2A}-adrenergic receptor and inhibition of adenylyl cyclase[64]. $G_z\alpha$ (41 kDa) is phosphorylated on Ser16 by p21-activated protein kinase (PAK)-1, an effector of the small GTPase Rac1. This decreases the inhibition by $\beta\gamma$ complexes and by RGSZ1, a G_z-selective GTPase-activating protein. A second phosphorylation site is Ser27, which is a target for protein kinase C[65,66]. $G_z\alpha$ is absent in MEG-01, DAMI and CHRF-288–11 cell lines, indicating that expression starts at a late stage of megakaryocyte maturation[28].

G_h

$G_h\alpha$ is a recently discovered G-protein subunit. Analysis by RT-PCR and immunoblotting reveals that $G_h\alpha$ is expressed in megakaryoblastic cell lines and platelets. Transfection studies in COS-7 show that the TPα -but not the TPβ receptor- couples to G_h, thereby taking part in thromboxane A_2 induced formation of inositolphosphate[67].

Cambridge Books Online © Cambridge University Press, 2009

Table 14.2. Small GTPases in platelets

Family	Member	Upstream activator/ regulator	Effector, localization	Refs
Ras-like	Ras	PAR1/3/4, TPα/β	Raf1, PtdIns 3-kinase, Rlf, $\alpha_{IIb}\beta_3$	77,79
		protein kinase C		78
	Rap1A	?		85
	Rap1B	Ca^{2+}, protein kinase C, integrin $\alpha_{IIb}\beta_3$, PtdIns 3-kinase	SERCA3, phospholipaseCγ1, Ral-GEF, plasma membrane, α granules	83,85,86,87,95,112
	Rap2B		αIIbβ3	98,103
	RalA	PAR1/3/4, paf-rec.,TPα/β,Rap1B	Cdc42GAP, dense granules, pl.membrane	105,109,110
	RalB		Cdc42–GEF(?)	77,105
Rho-like	RhoA	PAR1/3/4, Edg 2/4/7	Rho-kinase, PtdIns 3-kinase	112,114,115,142
		$G_{12}\alpha/G_{13}\alpha$		113
	Rac	PAR1/3/4	PtdIns 3-kinase, PtdIns 4–P 5– kinase	115,123,124
	Cdc42Hs	PAR1/3/4, $P2Y_1$	WAS protein	126,127
Rab	Rab1		α granules, lysosomes, pl. membrane, mitochondria	104,128
	Rab3B	protein kinase C	cytosol	128,129
	Rab4		lysosomes, plasma membrane, mitochondria	128
	Rab6	protein kinase C	α granules, plasma membrane	128,129
	Rab8	protein kinase C	α granules, plasma membrane	128,129
	Rab27A		dense granules	130,131
	Rab27B		α granules, plasma membrane	142

Notes:
Rab 3A absent in platelets[128].

Independent role of $\beta\gamma$ subunits

GDP-GTP replacement on α subunits triggers the dissociation of $\beta\gamma$ subunits, which act as independent signalling elements. Platelet $G_i\alpha_2$ and G_q appear major sources of free $\beta\gamma$. They contain predominantly γ5 but $\beta\gamma$ complexes from other cells equally effectively activate platelet phospholipases Cβ[68]. Targets are the phospholipases Cβ_2 and -β_{3a} in the membrane, and the -β_{3b} isoform in the cytosol[68]. The domain Glu574–Lys583 of phospholipase Cβ_2 appears to be the binding site for $\beta\gamma$[69,70]. Activation of phospholipase Cβ_2 and PtdIns 3-kinase-γ by $\beta_5\gamma_2$ has been reported[71]. In many cell types the β part is phosphorylated but in platelets this appears not to play a major role[70,72]. In rat platelets the γ_{11} isoform is present in relative excess[73].

Lysophosphatidic acid and sphingosine-1-phosphate bind to the Edg2/4/7 and Edg1/3/5 receptors respectively and are potent platelet activators. Subsequent signal induction is still poorly understood but in addition to G_q[74] and G_{12} /G_{13}[34,62] may involve G_i -derived $\beta\gamma$ subunits[75,76].

Small GTPases in platelets and megakaryocytes

Ras-like GTPases

Among the Ras-like GTPases identified in platelets so far is Ras itself. Subtype characterization is incomplete (Table 14.2). Ras is present at a concentration of about 0.5 µg/mg protein and activated in platelets stimulated with thrombin, SFLLRN peptide and thromboxane A_2[77,78]. Thus, activation pathways initiated by the PAR1 and TFα/β receptor couple to a Ras-GEF. In platelets stimulated with PMA, GDP-Ras is converted into GTP-Ras and it is therefore possible that protein kinase C functions as an activator of Ras[78]. The platelet inhibitor PGI_2 inhibits thrombin-induced Ras activation. Transfection of mutationally activated H-Ras in $\alpha_{IIb}\beta_3$ expressing CHO cells shows that the GTPase can act as a negative regulator of the integrin. Integrin inhibition was accompanied by activation of ERK1/2, which is a downstream element in the Ras pathway[79]. Also the β3-endonexin mediated activation of $\alpha_{IIb}\beta_3$ was blocked by H-Ras[80]. In contrast, R-Ras is an activator of integrins and appears to inhibit the H-Ras/Raf pathway[81]. Platelet p21rasGAP is tyrosine phosphorylated

Cambridge Books Online © Cambridge University Press, 2009

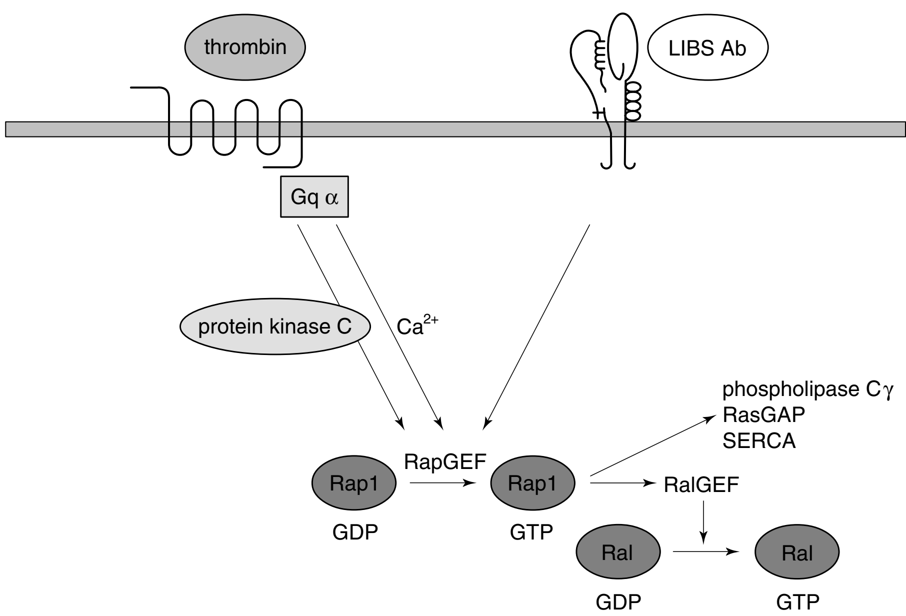


Fig. 14.4. Regulation of the GTPase Rap1. The inactive Rap1GDP is converted to active Rap1GTP by the RapGEF. Activation of Rap1 is biphasic: there is a first phase mediated by Ca^{2+} which is followed by a second phase mediated by protein kinase C. Also the monoclonal antibody LIBS-6, which activates integrin $\alpha_{IIb}\beta_3$ from the extracellular site, activates Rap1. Downstream targets of Rap1GTP are Ral, phospholipase Cγ (via RasGAP) and possibly the sarco/endoplasmatic reticulum Ca^{2+} ATPase, SERCA 3b.

and associates with the protein tyrosine kinases, Fyn, Lyn and Yes[82].

Rap1A is present in the cytosol, indicating that it lacks the posttranslational modification that enables membrane attachment[83]. In platelets Rap1A is tenfold lower than Rap1B, the latter accounting for 0.1% of protein content. In resting platelets, Rap1B is bound to membranes; upon platelet activation it translocates to the cytoskeleton[84]. Rap1B is activated in platelets stimulated by thrombin, PAR1 peptide, collagen, ADP, thromboxane A_2, platelet activating factor, low density lipoproteins and lysophosphatidic acid[85,86]. All activators raise cytosolic Ca^{2+}, which appears a crucial step in the initial phase of Rap1 activation (Fig. 14.4). This first activation phase is followed by a second phase which depends on protein kinase C and PtdIns 3-kinase[87]. Activation of integrin $\alpha_{IIb}\beta_3$ with a LIBS-6 antibody also induces Rap1 activation, indicating that the integrin signals to a Rap1GEF. Induction of platelet aggregation leads to inhibition of Rap1 activation. This downregulation correlates with Rap1 translocation to the cytoskeleton, possibly mediated by Rap1GAP, which also translocates to the cytoskeleton[88]. The contribution of Rap1GAP to Rap1 regulation appears minor, however, since upon platelet activation its activity hardly changes[89]. Two forms of Rap1GAP have been purified from platelet cytosol.

Rap1B activation is inhibited by prostacyclin but this effect appears independent of the phosphorylation induced by cAMP dependent protein kinase A. Rap1 is phosphorylated on Ser179 at the C-terminal end, which induces Rap1 translocation from plasma membranes to the cytosol[90]. Also cGMP-dependent protein kinase G phosphorylates Rap1. Thrombin equally activates Rap1 and phosphorylated Rap1[85]. Earlier work indicated that Rap1 might be involved in Ca^{2+} regulation in platelets[91,92]. Rap1 co-immunoprecipitated with the sarco/endoplasmic reticulum Ca^{2+}-ATPase isoform 3b (SERCA3b). The association was lost after protein kinase A-mediated phosphorylation of Rap1 on Ser179. The farnesylcysteine analogues farnesylthioacetic acid and N-acetyl-s-geranylgeranyl-L-cysteine inhibit methylation of prenylated and geranylgeranylated proteins respectively and decrease thrombin-induced increases in Ca^{2+} concentration[93,94]. Immunocytochemical localization shows that Rap1B in platelets and megakaryocytes is associated with the plasma membrane and the open canalicular system, as well as with α-granule membrane[95].

Phospholipase Cγ_1 is constitutively associated with the GAP for the Ras protein, p120RasGAP present in the cytosol. Thrombin triggers association of the complex to the membrane bound Rap1 thereby localizing phospholipase Cγ close to its substrates in the plasma membrane[83].

Cambridge Books Online © Cambridge University Press, 2009

Abnormalities in platelet Rap1 phosphorylation by protein kinase A have been reported in Grey Platelet Syndrome[96] and certain psychiatric disorders[97].

Platelets contain only the Rap2B isoform[98]. In contrast to Rap2A, which is farnesylated, Rap2B is geranylgeranylated similar to Rap1 proteins[99–101]. Upon platelet activation about 35% of total Rap2B translocates to the cytoskeleton in a process that critically depends on aggregation and actin polymerization. This translocation correlates with the cytoskeletal translocation of integrin $\alpha_{IIb}\beta_3$, suggesting that Rap2B functions in the regulation of fibrinogen binding and aggregation[83,84,102,103]. MEG-01 cells express Rap1A, Rap1B and Rap2B[104].

RalA and RalB have 55% sequence identity with Ras. Ral is abundantly present in human platelets[105,106] and post-translationally processed[107–109]. Ral is rapidly activated in platelets stimulated by thrombin, PAR1 peptide, platelet activating factor, thromboxane A_2[77,86]. ADP is a weak activator of Ral. The same activation is induced by Ca^{2+} ionophore, suggesting that the increase in cytosolic Ca^{2+} is a crucial step in Ral activation. In this respect, Ral activation and inhibition resembles that of Rap1B. This is the basis for the hypothesis that Rap1B is an upstream activator of the GEF for Ral[77,86] (Fig. 14.4). A Ral-GAP (34 kDa) has been found in the cytosol and particulate fraction[110].The only putative target of GTP-Ral is RLIP76, which is a GAP for Cdc42, a member of the Rho-like GTPases. If this interaction occurs in platelets, it would link the Rap1–Ral system to mechanisms that regulate cytoskeletal rearrangements. Ral is present in plasma membranes and especially dense granules[109], the latter suggesting that Ral functions in exocytosis[111]. Platelets contain RalA and RalB in approximately equal amounts[105]. Interestingly, RalA binds to phospholipase D, which has also been implicated in platelet secretion. This interaction is independent of the activity state of Ral, suggesting that Ral merely functions in targeting of PLD to the plasma membrane. MEG-01 cells express RalA[104].

Rho-like GTPases

Platelets contain Rho A, Rac1, Rac2 and Cdc42[112]. Platelet Rho is geranylgeranylated but nevertheless mainly cytosolic, possibly because a Rho-GDI prevents binding to the membrane. Thrombin stimulation induces translocation of Rho to the cytoskeleton.

Lysophosphatidic acid signals to the small GTPase Rho and regulation of actomyosin assembly[113]. This is the basis for platelet shape change induced by mildly oxidized low density lipoprotein, an important constituent of the atherosclerotic plaque[62]. *Clostridium botulinum* exoenzyme C3 ADP-ribosyl transferase[114] ribosylates Asn41 thereby preventing the interaction of the GTPase with downstream targets. This inhibitor has greatly facilitated the characterization of downstream pathways controlled by Rho. Thus, Rho inhibition abolishes activation of p85/p110 heterodimeric PtdIns 3-kinase[115]. Inhibition of Rho failed to affect inside-out signalling to integrin $\alpha_{IIb}\beta_3$ but decreased adhesion to fibrinogen and formation of vinculin-rich focal adhesions[116]. The Rho A inhibitor C3-transferase and the Rho-kinase inhibitor Y-27632 blocked lysophosphatidic acid induced myosin light chain phosphorylation, moesin phosphorylation and shape change[117]. A similar signalling pathway is initiated by sphingosine-1 phosphate, a second agonist of the lysophospholipid receptor family[118,119]. Rho signals via Rho-kinase inducing inhibition of myosin light chain phosphatase[120] (Fig. 14.5). Rho activity is regulated by a p190 RhoGAP which is associated with p120RasGAP[121]. Other GAPs of the Rho-like class are less specific for Rho and most of them have not yet been identified in platelets. Further control is carried out by Rho GDIs and at least one has been found in platelets[122]. MEG-01 cells express RhoA, Rac1, Rac2, Cdc42Hs[104].

Rac1 and Rac2 are present in platelets and translocate to the cytoskeleton upon platelet activation[122,123]. Rac activation by stimulating PAR receptors activates PtdIns 3-kinase[124]. Rac also activates PtdIns 4–P 5-kinase, resulting in formation of PtdIns 4,5 P_2 and actin filament barbed-end uncapping[123]. Another effector of Rac in platelets is γPAK, a member of the PAK65 class, which are Ser/Thr kinases involved in morphological changes. In other cells the Rac – PAK pathway signals to activation of the p38MAPkinase pathway, which in platelets signals to cytosolic phospholipase A_2 and formation of thromboxane A_2. This link is yet to be established in platelets. A RacGEF in platelets is Vav. Vav is phosphorylated in platelets stimulated with strong agonists and a target of outside-in signalling through integrins during platelet adhesion[125].

Cdc42 is geranylgeranylated and shows 50% identity with Rho and about 30% identity with the Ras-like proteins. Platelet Cdc42 is present in plasma membranes and in the cytosol[106,112]. Despite its posttranslationally modified C-terminus, it is present in the cytosol probably in a complex with a RhoGDI. Platelet stimulation with thrombin or ADP induces translocation to the cytoskeleton, which appears to occur during the second phase of a biphasic aggregation response[123]. An effector of Cdc42 is the Wiskott Aldrich syndrome protein, which is deficient in patients suffering from Wiskott Aldrich Syndrome[126,127]. These platelets have an abnormal morphology due to a defect in the actin skeleton. A Cdc42GAP is present in the cytosol and in membranes.

Cambridge Books Online © Cambridge University Press, 2009

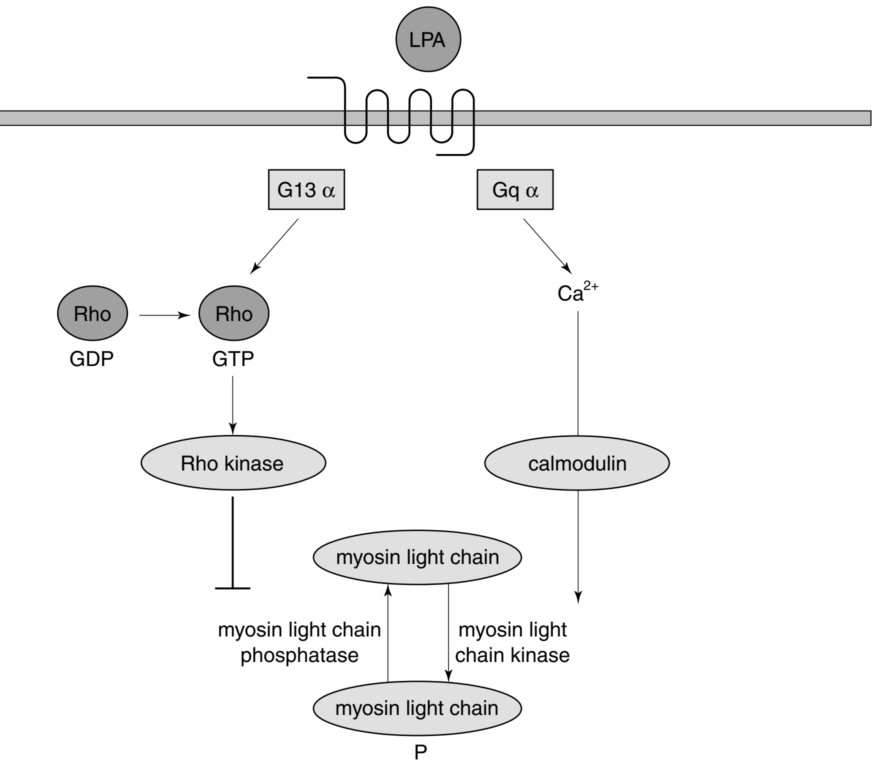


Fig. 14.5. Role of Rho in platelet shape change. Binding of lysophosphatidic acid (LPA) to its receptor activates G_{13} and G_q. G_{13} signalling involves activation of RhoGDP to RhoGTP and subsequent activation of Rho-kinase, which is an inhibitor of myosin light chain phosphatase. G_q signalling leads to a Ca^{2+} increase and together with calmodulin to activation of myosin light chain kinase. The result of both routes is phosphorylation of myosin light chain, actin–myosin interaction and cytoskeleton reorganization during shape change (adapted from[24]).

Rab GTPases

Rab proteins are involved in the docking of transport vesicles with their target compartments. They are doubly geranylgeranylated and about 30% homologous with Ras. So far seven members of this class have been found in platelets[104,128]. Rab3B is present in the cytosol whereas as Rab6 and Rab8 are found in plasma membranes and α-granules[128]. Thrombin activation of platelets leads to protein kinase C dependent phosphorylation of Rab3B, -6 and –8 and translocation of Rab6 to the cytosol[128,129]. Mice homozygous for the mutation gunmetal (gm) have prolonged bleeding, thrombocytopenia, and reduced platelet α- and δ-granule contents[130]. This is caused by a G-A substitution in Rab geranylgeranyl transferase, which catalyzes the attachment of geranylgeranyl groups to Rab. This results in a severe reduction in enzyme activity, membrane association of Rab27, and platelet formation[131]. MEG-01 cells express Rab3B, Rab6 but not Rab4[104].

Acknowledgements

The author thanks Dr G.van Willigen for helpful comments. JWNA is supported by the Netherlands Thrombosis Foundation.

References

1 Neer EJ. Heterotrimeric G proteins: organizers of transmembrane signals. *Cell* 1995; 80: 249–257.
2 Selbie LA, Hill SJ. G protein-coupled-receptor cross-talk: the fine-tuning of multiple receptor-signalling pathways. *Trends Pharmacol Sci* 1998; 19: 87–93.
3 Brass LF, Manning DR, Cichowski K, Abrams CS. Signalling through G proteins in platelets: to the integrins and beyond. *Thromb Haemost* 1997; 78: 581–589.
4 Freissmuth M, Waldhoer M, Bofill-Cardona E, Nanoff C. G protein antagonists. *Trends Pharmacol Sci* 1999; 20: 237–245.

Cambridge Books Online © Cambridge University Press, 2009

5 Offermanns S. New insights into the in vivo function of heterotrimeric G-proteins through gene deletion studies. *Naunyn Schmiedebergs Arch Pharmacol* 1999; 360: 5–13.
6 Vale RD. Switches, latches, and amplifiers: common themes of G proteins and molecular motors. *J Cell Biol* 1996; 135: 291–302.
7 van Willigen G, Nieuwland R, Nurnberg B, Gorter G, Akkerman JW. Negative regulation of the platelet Na+/H+ exchanger by trimeric G-proteins. *Eur J Biochem* 2000; 267: 7102–7108.
8 Meng J, Glick JL, Polakis P, Casey PJ. Functional interaction between Galpha(z) and Rap1GAP suggests a novel form of cellular cross-talk. *J Biol Chem* 1999; 274: 36663–36669.
9 Amatruda TT, III, Steele DA, Slepak VZ, Simon MI. G alpha 16, a G protein alpha subunit specifically expressed in hematopoietic cells. *Proc Natl Acad Sci USA* 1991; 88: 5587–5591.
10 Qian NX, Russell M, Buhl AM, Johnson GL. Expression of GTPase-deficient G alpha 16 inhibits Swiss 3T3 cell growth. *J Biol Chem* 1994; 269: 17417–17423.
11 Offermanns S, Laugwitz KL, Spicher K, Schultz G. G proteins of the G12 family are activated via thromboxane A2 and thrombin receptors in human platelets. *Proc Natl Acad Sci USA* 1994; 91: 504–508.
12 Rebollo A, Martinez A. Ras proteins: recent advances and new functions. *Blood* 1999; 94: 2971–2980.
13 Kroner C, Eybrechts K, Akkerman JW. Dual regulation of platelet protein kinase B. *J Biol Chem* 2000; 275: 27790–27798.
14 Bos JL, Franke B, M'Rabet L, Reedquist K, Zwartkruis F. In search of a function for the Ras-like GTPase Rap1. *FEBS Lett* 1997; 410: 59–62.
15 Bos JL. All in the family? New insights and questions regarding interconnectivity of Ras, Rap1 and Ral. *EMBO J* 1998; 17: 6776–6782.
16 de Rooij J, Zwartkruis FJ, Verheijen MH et al. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* 1998; 396: 474–477.
17 Tsukamoto N, Hattori M, Yang H, Bos JL, Minato N. Rap1 GTPase-activating protein SPA-1 negatively regulates cell adhesion. *J Biol Chem* 1999; 274: 18463–18469.
18 Bishop AL, Hall A. Rho GTPases and their effector proteins. *Biochem J* 2000; 348: 241–255.
19 Ridley A. Rho GTPases. Integrating integrin signalling. *J Cell Biol* 2000; 150: F107–F109.
20 Levy-Toledano S. Platelet signal transduction pathways: could we organize them into a 'hierarchy'? *Haemostasis* 1999; 29: 4–15.
21 Brass LF, Hoxie JA, Manning DR. Signalling through G proteins and G protein-coupled receptors during platelet activation. *Thromb Haemost* 1993; 70: 217–223.
22 Manning DR, Brass LF. The role of GTP-binding proteins in platelet activation. *Thromb Haemost* 1991; 66: 393–399.
23 Akkerman JW, van Willigen G. Platelet activation via trimeric GTP-binding proteins. *Haemostasis* 1996; 26 Suppl 4: 199–209.
24 Offermanns S. The role of heterotrimeric G proteins in platelet activation. *Biol Chem* 2000; 381: 389–396.
25 van Willigen G, Donath J, Lapetina EG, Akkerman JW. Identification of alpha-subunits of trimeric GTP-binding proteins in human platelets by RT-PCR. *Biochem Biophys Res Commun* 1995; 214: 254–262.
26 Watson EL. GTP-binding proteins and regulated exocytosis. *Crit Rev Oral Biol Med* 1999; 10: 284–306.
27 Ferreira P, Meyer I, Mollner S, Frank R, Pfeuffer T. Selective formation of Gsalpha-MHC I complexes after desensitization of human platelets with iloprost. *Eur J Biochem* 1999; 259: 167–174.
28 van der Vuurst H, van Willigen G, van Spronsen A, Hendriks M, Donath J, Akkerman JW. Signal transduction through trimeric G proteins in megakaryoblastic cell lines. *Arterioscler Thromb Vasc Biol* 1997; 17: 1830–1836.
29 van der Vuurst H, Hendriks M, Lapetina EG, van Willigen G, Akkerman JW. Maturation of megakaryoblastic cells is accompanied by upregulation of G(s)alpha-L subtype and increased cAMP accumulation. *Thromb Haemost* 1998; 79: 1014–1020.
30 Mitchell PB, Manji HK, Chen G et al. High levels of Gs alpha in platelets of euthymic patients with bipolar affective disorder. *Am J Psychiatry* 1997; 154: 218–223.
31 Mooney JJ, Samson JA, McHale NL et al. Signal transduction by platelet adenylate cyclase: alterations in depressed patients may reflect impairment in the coordinated integration of cellular signals (coincidence detection). *Biol Psychiatry* 1998; 43: 574–583.
32 Ohlmann P, Laugwitz KL, Nurnberg B et al. The human platelet ADP receptor activates Gi2 proteins. *Biochem J* 1995; 312: 775–779.
33 Ohlmann P, Eckly A, Freund M, Cazenave JP, Offermanns S, Gachet C. ADP induces partial platelet aggregation without shape change and potentiates collagen-induced aggregation in the absence of Galphaq. *Blood* 2000; 96: 2134–2139.
34 Essler M, Retzer M, Bauer M, Zangl KJ, Tigyi G, Siess W. Stimulation of platelets and endothelial cells by mildly oxidized LDL proceeds through activation of lysophosphatidic acid receptors and the Rho/Rho-kinase pathway. Inhibition by lovastatin. *Ann NY Acad Sci* 2000; 905: 282–286.
35 Giesberts AN, van Willigen G, Lapetina EG, Akkerman JW. Regulation of platelet glycoprotein IIb/IIIa (integrin alpha IIB beta 3) function via the thrombin receptor. *Biochem J* 1995; 309: 613–620.
36 Nakanishi-Matsui M, Zheng YW, Sulciner DJ, Weiss EJ, Ludeman MJ, Coughlin SR. PAR3 is a cofactor for PAR4 activation by thrombin. *Nature* 2000; 404: 609–613.
37 Jantzen HM, Milstone DS, Gousset L, Conley PB, Mosher DF. Impaired ADP-induced platelet activation in Galpha i2-deficient mice. *Blood* 2000; 94: 2749–2750.
38 Leon C, Hechler B, Freund M et al. Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. *J Clin Invest* 1999; 104: 1731–1737.
39 Hollopeter G, Jantzen H-M, Vincent D et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* 2001; 409: 202–207.

Cambridge Books Online © Cambridge University Press, 2009

40 Jantzen HM, Gousset L, Bhaskar V, Vincent D, Tai A, Reynolds EE, Conley PB. Evidence for two distinct G-protein-coupled ADP receptors mediating platelet activation. *Thromb Haemost* 1999; 81: 111–117.

41 Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. *Proc Natl Acad Sci USA* 1998; 95: 8070–8074.

42 Nieuwland R, Wijburg OL, van Willigen G, Akkerman JW. Alpha 2A-adrenergic receptors activate protein kinase C in human platelets via a pertussis toxin-sensitive G-protein. *FEBS Lett* 1994; 339: 79–83.

43 Torti M, Crouch MF, Lapetina EG. Epinephrine induces association of pp60src with Gi alpha in human platelets. *Biochem Biophys Res Commun* 1992; 186: 440–447.

44 Frazier WA, Gao AG, Dimitry J et al. The thrombospondin receptor integrin-associated protein (CD47) functionally couples to heterotrimeric Gi. *J Biol Chem* 1999; 274: 8554–8560.

45 Siffert W, Jakobs KH, Akkerman JW. Sodium fluoride prevents receptor- and protein kinase C-mediated activation of the human platelet Na+/H+ exchanger without inhibiting its basic pHi-regulating activity. *J Biol Chem* 1990; 265: 15441–15448.

46 Li RY, Gaits F, Ragab A, Ragab-Thomas JM, Chap H. Tyrosine phosphorylation of an SH2-containing protein tyrosine phosphatase is coupled to platelet thrombin receptor via a pertussis toxin-sensitive heterotrimeric G-protein. *EMBO J* 1995; 14: 2519–2526.

47 Garcia-Sevilla JA, Walzer C, Busquets X, Escriba PV, Balant L, Guimon J. Density of guanine nucleotide-binding proteins in platelets of patients with major depression: increased abundance of the G alpha i2 subunit and down-regulation by antidepressant drug treatment. *Biol Psychiatry* 1997; 42: 704–712.

48 Gurguis GN, Vo SP, Griffith JM, Rush AJ. Platelet alpha2A-adrenoceptor function in major depression: Gi coupling, effects of imipramine and relationship to treatment outcome. *Psychiatry Res* 1999; 89: 73–95.

49 Marcil J, Schiffrin EL, Anand-Srivastava MB. Aberrant adenylyl cyclase/cAMP signal transduction and G protein levels in platelets from hypertensive patients improve with antihypertensive drug therapy. *Hypertension* 1996; 28: 83–90.

50 Offermanns S, Toombs CF, Hu YH, Simon MI. Defective platelet activation in G alpha(q)-deficient mice. *Nature* 1997; 389: 183–186.

51 Pulcinelli FM, Ciampa MT, Favilla M, Pignatelli P, Riondino S, Gazzaniga PP. Concomitant activation of Gi protein-coupled receptor and protein kinase C or phospholipase C is required for platelet aggregation. *FEBS Lett* 1999; 460: 37–40.

52 Shah BH, Siddiqui A, Qureshi KA et al. Co-activation of Gi and Gq proteins exerts synergistic effect on human platelet aggregation through activation of phospholipase C and Ca^{2+} signalling pathways. *Exp Mol Med* 1999; 31: 42–46.

53 Paul BZ, Jin J, Kunapuli SP. Molecular mechanism of thromboxane A(2)-induced platelet aggregation. Essential role for p2t(ac) and alpha(2a) receptors. *J Biol Chem* 1999; 274: 29108–29114.

54 Klages B, Brandt U, Simon MI, Schultz G, Offermanns S. Activation of G12/G13 results in shape change and Rho/Rho-kinase-mediated myosin light chain phosphorylation in mouse platelets. *J Cell Biol* 1999; 144: 745–754.

55 Walsh MT, Foley JF, Kinsella BT. The alpha, but not the beta, isoform of the human thromboxane A2 receptor is a target for prostacyclin-mediated desensitization. *J Biol Chem* 2000; 275: 20412–20423.

56 Gabbeta J, Yang X, Kowalska MA, Sun L, Dhanasekaran N, Rao AK. Platelet signal transduction defect with Galpha subunit dysfunction and diminished Galphaq in a patient with abnormal platelet responses. *Proc Natl Acad Sci USA* 1997; 94: 8750–8755.

57 Israels SJ, Cheang T, Roberston C, McMillan-Ward EM, McNicol A. Impaired signal transduction in neonatal platelets. *Pediatr Res* 1999; 45: 687–691.

58 Johnson GJ, Leis LA, Dunlop PC. Thromboxane-insensitive dog platelets have impaired activation of phospholipase C due to receptor-linked G protein dysfunction. *J Clin Invest* 1993; 92: 2469–2479.

59 Offermanns S, Hu YH, Simon MI. G alpha12 and g alpha13 are phosphorylated during platelet activation. *J Biol Chem* 1996; 271: 26044–26048.

60 Djellas Y, Manganello JM, Antonakis K, Le Breton GC. Identification of G alpha13 as one of the G-proteins that couple to human platelet thromboxane A2 receptors. *J Biol Chem* 1999; 274: 14325–14330.

61 Manganello JM, Djellas Y, Borg C, Antonakis K, Le Breton GC. Cyclic AMP-dependent phosphorylation of thromboxane A(2) receptor-associated G alpha(13). *J Biol Chem* 1999; 274: 28003–28010.

62 Maschberger P, Bauer M, Baumann-Siemons J et al. Mildly oxidized low density lipoprotein rapidly stimulates via activation of the lysophosphatidic acid receptor Src family and Syk tyrosine kinases and Ca^{2+} influx in human platelets. *J Biol Chem* 2000; 275: 19159–19166.

63 Tenailleau S, Corre I, Hermouet S. Specific expression of heterotrimeric G proteins G12 and G16 during human myeloid differentiation. *Exp Hematol* 1997; 25: 927–934.

64 Yang J, Wu J, Kowalska MA et al. Loss of signalling through the G protein, Gz, results in abnormal platelet activation and altered responses to psychoactive drugs. *Proc Natl Acad Sci USA* 2000; 97: 9984–9989.

65 Wang J, Frost JA, Cobb MH, Ross EM. Reciprocal signalling between heterotrimeric G proteins and the p21-stimulated protein kinase. *J Biol Chem* 1999; 274: 31641–31647.

66 Lounsbury KM, Casey PJ, Brass LF, Manning DR. Phosphorylation of Gz in human platelets. Selectivity and site of modification. *J Biol Chem* 1991; 266: 22051–22056.

67 Vezza R, Habib A, FitzGerald GA. Differential signalling by the thromboxane receptor isoforms via the novel GTP-binding protein, Gh. *J Biol Chem* 1999; 274: 12774–12779.

68 Lee CH, Park D, Wu D, Rhee SG, Simon MI. Members of the Gq alpha subunit gene family activate phospholipase C beta isozymes. *J Biol Chem* 1992; 267: 16044–16047.

Cambridge Books Online © Cambridge University Press, 2009

69 Sankaran B, Osterhout J, Wu D, Smrcka AV. Identification of a structural element in phospholipase C beta2 that interacts with G protein betagamma subunits. *J Biol Chem* 1998; 273: 7148–7154.

70 Banno Y, Asano T, Nozawa Y. Stimulation by G protein betagamma subunits of phospholipase C beta isoforms in human platelets. *Thromb Haemost* 1998; 79: 1008–1013.

71 Maier U, Babich A, Macrez N et al. Gbeta 5gamma 2 is a highly selective activator of phospholipid-dependent enzymes. *J Biol Chem* 2000; 275: 13746–13754.

72 Nurnberg B, Harhammer R, Exner T, Schulze RA, Wieland T. Species- and tissue-dependent diversity of G-protein beta subunit phosphorylation: evidence for a cofactor. *Biochem J* 1996; 318: 717–722.

73 Morishita R, Ueda H, Kato K, Asano T. Identification of two forms of the gamma subunit of G protein, gamma10 and gamma11, in bovine lung and their tissue distribution in the rat. *FEBS Lett* 1998; 428: 85–88.

74 Nietgen GW, Durieux ME. Intercellular signalling by lysophosphatidate. *Cell Adhes Commun* 1998; 5: 221–235.

75 Hla T, Lee MJ, Ancellin N et al. Sphingosine-1-phosphate signalling via the EDG-1 family of G-protein-coupled receptors. *Ann N Y Acad Sci* 2000; 905: 16–24.

76 Goetzl EJ, An S. Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. *FASEB J* 1998; 12: 1589–1598.

77 Wolthuis RM, Franke B, van Triest M, Bauer B, Cool RH, Camonis JH, Akkerman JW, Bos JL. Activation of the small GTPase Ral in platelets. *Mol Cell Biol* 1998; 18: 2486–2491.

78 Shock DD, He K, Wencel-Drake JD, Parise LV. Ras activation in platelets after stimulation of the thrombin receptor, thromboxane A2 receptor or protein kinase C. *Biochem J* 1997; 321: 525–530.

79 Hughes PE, Renshaw MW, Pfaff M et al. Suppression of integrin activation: a novel function of a Ras/Raf-initiated MAP kinase pathway. *Cell* 1997; 88: 521–530.

80 Kashiwagi H, Schwartz MA, Eigenthaler M, Davis KA, Ginsberg MH, Shattil SJ. Affinity modulation of platelet integrin alphaIIbbeta3 by beta3-endonexin, a selective binding partner of the beta3 integrin cytoplasmic tail. *J Cell Biol* 1997; 137: 1433–1443.

81 Sethi T, Ginsberg MH, Downward J, Hughes PE. The small GTP-binding protein R-Ras can influence integrin activation by antagonizing a Ras/Raf-initiated integrin suppression pathway. *Mol Biol Cell* 1999; 10: 1799–1809.

82 Cichowski K, McCormick F, Brugge JS. p21rasGAP association with Fyn, Lyn, and Yes in thrombin-activated platelets. *J Biol Chem* 1992; 267: 5025–5028.

83 Torti M, Lapetina EG. Role of rap1B and p21ras GTPase-activating protein in the regulation of phospholipase C-gamma 1 in human platelets. *Proc Natl Acad Sci USA* 1992; 89: 7796–7800.

84 Torti M, Bertoni A, Canobbio I, Sinigaglia F, Lapetina EG, Balduini C. Rap1B and Rap2B translocation to the cytoskeleton by von Willebrand factor involves FcgammaII receptor-mediated protein tyrosine phosphorylation. *J Biol Chem* 1999; 274: 13690–13697.

85 Franke B, Akkerman JW, Bos JL. Rapid Ca2+-mediated activation of Rap1 in human platelets. *EMBO J* 1997; 16: 252–259.

86 Hackeng CM, Franke B, Relou IA et al. Low-density lipoprotein activates the small GTPases Rap1 and Ral in human platelets. *Biochem J* 2000; 349: 231–238.

87 Franke B, van Triest M, de Bruijn KM, van Willigen G, Nieuwenhuis HK, Negrier C, Akkerman JW, Bos JL. Sequential regulation of the small GTPase Rap1 in human platelets. *Mol Cell Biol* 2000; 20: 779–785.

88 Fischer TH, Gatling MN, McCormick F, Duffy CM, White GC. Incorporation of Rap 1b into the platelet cytoskeleton is dependent on thrombin activation and extracellular calcium. *J Biol Chem* 1994; 269: 17257–17261.

89 Marti KB, Lapetina EG. Epinephrine suppresses rap1B.GAP-activated GTPase activity in human platelets. *Proc Natl Acad Sci USA* 1992; 89: 2784–2788.

90 Siess W, Winegar DA, Lapetina EG. Rap1–B is phosphorylated by protein kinase A in intact human platelets. *Biochem Biophys Res Commun* 1990; 170: 944–950.

91 Lacabaratz-Porret C, Corvazier E, Kovacs T et al. Platelet sarco/endoplasmic reticulum Ca2+ATPase isoform 3b and Rap 1b: interrelation and regulation in physiopathology. *Biochem J* 1998; 332: 173–181.

92 Magnier C, Corvazier E, Aumont MC, Le Jemtel TH, Enouf J. Relationship between Rap1 protein phosphorylation and regulation of Ca2+ transport in platelets: a new approach. *Biochem J* 1995; 310: 469–475.

93 Rosado JA, Sage SO. Farnesylcysteine analogues inhibit store-regulated Ca^{2+} entry in human platelets: evidence for involvement of small GTP-binding proteins and actin cytoskeleton. *Biochem J* 2000; 347: 183–192.

94 Rosado JA, Sage SO. Regulation of plasma membrane Ca^{2+}-ATPase by small GTPases and phosphoinositides in human platelets. *J Biol Chem* 2000; 275: 19529–19535.

95 Berger G, Quarck R, Tenza D, Levy-Toledano S, de Gunzburg J, Cramer EM. Ultrastructural localization of the small GTP-binding protein Rap1 in human platelets and megakaryocytes. *Br J Haematol* 1994; 88: 372–382.

96 Enouf J, Corvazier E, Papp B et al. Abnormal cAMP-induced phosphorylation of rap 1 protein in grey platelet syndrome platelets. *Br J Haematol* 1994; 86: 338–346.

97 Perez J, Tardito D, Mori S, Racagni G, Smeraldi E, Zanardi R. Altered Rap1 endogenous phosphorylation and levels in platelets from patients with bipolar disorder. *J Psychiatr Res* 2000; 34: 99–104.

98 Torti M, Bertoni A, Canobbio I, Sinigaglia F, Lapetina EG, Balduini C. Interaction of the low-molecular-weight GTP-binding protein rap2 with the platelet cytoskeleton is mediated by direct binding to the actin filaments. *J Cell Biochem* 1999; 75: 675–685.

99 Farrell FX, Lapetina EG. Partial purification of a GTPase-activating protein for rap2b from bovine brain membranes. *Biochem Biophys Res Commun* 1992; 189: 717–721.

100 Farrell FX, Yamamoto K, Lapetina EG. Prenyl group identifi-

Cambridge Books Online © Cambridge University Press, 2009

cation of rap2 proteins: a ras superfamily member other than ras that is farnesylated. *Biochem J* 1993; 289: 349–355.

101 Vedia L, Ohmstede CA, Lapetina EG. Properties of the exchange rate of guanine nucleotides to the novel rap-2B protein. *Biochem Biophys Res Commun* 1990; 171: 319–324.

102 Torti M, Ramaschi G, Sinigaglia F, Lapetina EG, Balduini C. Glycoprotein IIb–IIIa and the translocation of Rap2B to the platelet cytoskeleton. *Proc Natl Acad Sci USA* 1994; 91: 4239–4243.

103 Torti M, Ramaschi G, Sinigaglia F, Lapetina EG, Balduini C. Association of the low molecular weight GTP-binding protein rap2B with the cytoskeleton during platelet aggregation. *Proc Natl Acad Sci USA* 1993; 90: 7553–7557.

104 Nagata K, Okano Y, Nozawa Y. Differential expression of low Mr GTP-binding proteins in human megakaryoblastic leukemia cell line, MEG-01, and their possible involvement in the differentiation process. *Thromb Haemost* 1997; 77: 368–375.

105 Jilkina O, Bhullar RP. Generation of antibodies specific for the RalA and RalB GTP-binding proteins and determination of their concentration and distribution in human platelets. *Biochim Biophys Acta* 1996; 1314: 157–166.

106 Polakis PG, Weber RF, Nevins B, Didsbury JR, Evans T, Snyderman R. Identification of the ral and rac1 gene products, low molecular mass GTP-binding proteins from human platelets. *J Biol Chem* 1989; 264: 16383–16389.

107 Bhullar RP, Yang S. Immunodetection of ralA and ralB GTP-binding proteins in various rat tissues and platelets. *Mol Cell Biochem* 1998; 179: 49–55.

108 Bhullar RP, Chardin P, Haslam RJ. Identification of multiple ral gene products in human platelets that account for some but not all of the platelet Gn-proteins. *FEBS Lett* 1990; 260: 48–52.

109 Mark BL, Jilkina O, Bhullar RP. Association of Ral GTP-binding protein with human platelet dense granules. *Biochem Biophys Res Commun* 1996; 225: 40–46.

110 Bhullar RP, Seneviratne HD. Characterization of human platelet GTPase activating protein for the Ral GTP-binding protein. *Biochim Biophys Acta* 1996; 1311: 181–188.

111 Bhullar RP, Yang S. Immunodetection of ralA and ralB GTP-binding proteins in various rat tissues and platelets. *Mol Cell Biochem* 1998; 179: 49–55.

112 Dash D, Aepfelbacher M, Siess W. The association of pp125FAK, pp60Src, CDC42Hs and Rap1B with the cytoskeleton of aggregated platelets is a reversible process regulated by calcium. *FEBS Lett* 1995; 363: 231–234.

113 Moolenaar WH. Bioactive lysophospholipids and their G protein-coupled receptors. *Exp Cell Res* 1999; 253: 230–238.

114 Nemoto Y, Namba T, Teru-uchi T, Ushikubi F, Morii N, Narumiya S. A rho gene product in human blood platelets. I. Identification of the platelet substrate for botulinum C3 ADP-ribosyltransferase as rhoA protein. *J Biol Chem* 1992; 267: 20916–20920.

115 Zhang J, King WG, Dillon S, Hall A, Feig L, Rittenhouse SE. Activation of platelet phosphatidylinositide 3-kinase requires the small GTP-binding protein Rho. *J Biol Chem* 1993; 268: 22251–22254.

116 Leng L, Kashiwagi H, Ren XD, Shattil SJ. RhoA and the function of platelet integrin alphaIIbbeta3. *Blood* 1998; 91: 4206–4215.

117 Retzer M, Siess W, Essler M. Mildly oxidised low density lipoprotein induces platelet shape change via Rho-kinase-dependent phosphorylation of myosin light chain and moesin. *FEBS Lett* 2000; 466: 70–74.

118 Moolenaar WH. Lysophosphatidic acid signalling. *Curr Opin Cell Biol* 1995; 7: 203–210.

119 Zhang Q, Peyruchaud O, French KJ, Magnusson MK, Mosher DF. Sphingosine 1-phosphate stimulates fibronectin matrix assembly through a Rho-dependent signal pathway. *Blood* 1999; 93: 2984–2990.

120 Suzuki Y, Yamamoto M, Wada H et al. Agonist-induced regulation of myosin phosphatase activity in human platelets through activation of Rho-kinase. *Blood* 1999; 93: 3408–3417.

121 Cichowski K, McCormick F, Brugge JS. p21rasGAP association with Fyn, Lyn, and Yes in thrombin-activated platelets. *J Biol Chem* 1992; 267: 5025–5028.

122 Dash D, Aepfelbacher M, Siess W. Integrin alpha IIb beta 3-mediated translocation of CDC42Hs to the cytoskeleton in stimulated human platelets. *J Biol Chem* 1995; 270: 17321–17326.

123 Azim AC, Barkalow K, Chou J, Hartwig JH. Activation of the small GTPases, rac and cdc42, after ligation of the platelet PAR-1 receptor. *Blood* 2000; 95: 959–964.

124 Bokoch GM, Vlahos CJ, Wang Y, Knaus UG, Traynor-Kaplan AE. Rac GTPase interacts specifically with phosphatidylinositol 3-kinase. *Biochem J* 1996; 315: 775–779.

125 Cichowski K, Brugge JS, Brass LF. Thrombin receptor activation and integrin engagement stimulate tyrosine phosphorylation of the proto-oncogene product, p95vav, in platelets. *J Biol Chem* 1996; 271: 7544–7550.

126 Snapper SB, Rosen FS. The Wiskott-Aldrich syndrome protein (WASP): roles in signalling and cytoskeletal organization. *Annu Rev Immunol* 1999; 17: 905–929.

127 Kolluri R, Tolias KF, Carpenter CL, Rosen FS, Kirchhausen T. Direct interaction of the Wiskott-Aldrich syndrome protein with the GTPase Cdc42. *Proc Natl Acad Sci USA* 1996; 93: 5615–5618.

128 Karniguian A, Zahraoui A, Tavitian A. Identification of small GTP-binding rab proteins in human platelets: thrombin-induced phosphorylation of rab3B, rab6, and rab8 proteins. *Proc Natl Acad Sci USA* 1993; 90: 7647–7651.

129 Fitzgerald ML, Reed GL. Rab6 is phosphorylated in thrombin-activated platelets by a protein kinase C-dependent mechanism: effects on GTP/GDP binding and cellular distribution. *Biochem J* 1999; 342: 353–360.

130 Wilson SM, Yip R, Swing DA et al. A mutation in Rab27a causes the vesicle transport defects observed in ashen mice. *Proc Natl Acad Sci USA* 2000; 97: 7933–7938.

131 Detter JC, Zhang Q, Mules EH et al. Rab geranylgeranyl transferase alpha mutation in the gunmetal mouse reduces Rab prenylation and platelet synthesis. *Proc Natl Acad Sci USA* 2000; 97: 4144–4149.

132 Wilkie TM, Scherle PA, Strathmann MP, Slepak VZ, Simon MI. Characterization of G-protein alpha subunits in the Gq class:

Cambridge Books Online © Cambridge University Press, 2009

expression in murine tissues and in stromal and hematopoietic cell lines. *Proc Natl Acad Sci USA* 1991; 88: 10049–10053.

133 Johnson GJ, Leis LA, Dunlop PC. Specificity of G alpha q and G alpha 11 gene expression in platelets and erythrocytes. Expressions of cellular differentiation and species differences. *Biochem J* 1996; 318: 1023–1031.

134 Ashby B. Prostaglandin regulation of cAMP metabolism in human platelets. *Platelets* 1990; 1: 11–20.

135 Brass LF, Molino M. Protease-activated G protein-coupled receptors on human platelets and endothelial cells. *Thromb Haemost* 1997; 78: 234–241.

136 Siess W. Molecular mechanisms of platelet activation. *Physiol Rev* 1989; 69: 58–178.

137 Hung DT, Wong YH, Vu TK, Coughlin SR. The cloned platelet thrombin receptor couples to at least two distinct effectors to stimulate phosphoinositide hydrolysis and inhibit adenylyl cyclase. *J Biol Chem* 1992; 267: 20831–20834.

138 Benka ML, Lee M, Wang GR et al. The thrombin receptor in human platelets is coupled to a GTP binding protein of the G alpha q family. *FEBS Lett* 1995; 363: 49–52.

139 Thibonnier M, Goraya T, Berti-Mattera L. G protein coupling of human platelet V1 vascular vasopressin receptors. *Am J Physiol* 1993; 264: C1336–C1344.

140 Becker KP, Garnovskaya M, Gettys T, Halushka PV. Coupling of thromboxane A2 receptor isoforms to Galpha13: effects on ligand binding and signalling. *Biochim Biophys Acta* 1999; 1450: 288–296.

141 Carlson KE, Brass LF, Manning DR. Thrombin and phorbol esters cause the selective phosphorylation of a G protein other than Gi in human platelets. *J Biol Chem* 1989; 264: 13298–13305.

142 Chen D, Guo J, Miki T, Tachibana M, Gahl WA. Molecular cloning and characterization of rab27a and rab27b, novel human rab proteins shared by melanocytes and platelets. *Biochem Mol Med* 1997; 60: 27–37.

Cambridge Books Online © Cambridge University Press, 2009

Platelet phospholipases A_2

Michael H. Gelb[1], Carine M. Mounier[2], Ying Hefner[1] and Steve P. Watson[3]

[1]*Departments of Chemistry and Biochemistry, University of Washington, Seattle, USA*
[2]*Département de Biologie, Université de Cergy-Pontoise, France*
[3]*Department of Pharmacology, University of Oxford, UK*

Introduction

The major physiological role of platelets is in the cessation of bleeding following damage to the vasculature. Ordinarily, platelets circulate within intact blood vessels in a quiescent state, but undergo extremely rapid and powerful activation upon exposure to the subendothelial matrix leading to formation of a platelet aggregate or vascular plug. This rapid response is achieved by the stimulatory action of a range of diverse agonists including extracellular matrix proteins, e.g. collagen, and products of the coagulation cascade, e.g. thrombin. Activation is reinforced through release of agonists from platelet granules, notably ADP, and liberation of thromboxane A_2 (TxA_2), the major product of the metabolism of arachidonic acid (AA) in the platelet. The positive feedback action of ADP and TxA_2 is of direct clinical relevance. The Antiplatelet Trialists' Collaboration[1] published a summary of 20 randomized trials in 1988, concluding that antiplatelet therapy significantly reduces (by ~ 25%) the risk of cardiovascular death, non-fatal myocardial infarction and nonfatal stroke in patients with unstable angina or a past history of heart attack, transient ischemic attack, or stroke[2,3]. A follow-up report by the same group, confirmed the efficacy of platelet inhibition in a broader spectrum of pathological conditions[4]. More recently, the CAPRIE study has shown that the ADP receptor antagonist, clopidogrel, and aspirin have a similar therapeutic benefit in individuals with a history of thrombotic disease[5]. Aspirin, however, remains the drug of choice in the majority of cases primarily for reasons of cost.

The availability of AA is the rate-limiting step in the formation of TxA_2 in platelets. As will be presented in detail below, it is now well established that AA is released from membrane phospholipids in activated platelets mainly by the enzyme phospholipase A_2 (PLA_2) activity. PLA_2 hydrolyses the *sn*-2 ester of glycerophospholipids to release a free fatty acid (AA and others) and a lysophospholipid. PLA_2 would also be a very good target for development of antithrombotics. This potential is unlikely to be exploited because of the proven efficacy of aspirin as an antiplatelet agent. Aspirin is an irreversible inhibitor of platelet cyclooxygenase-1 (COX-1), and, because platelets lack a nucleus, the enzyme cannot be replenished within the lifetime of the cell. As a consequence, only a low dosage of aspirin is required to achieve full blockade of TxA_2 formation in platelets, whereas other cells can generate new enzyme within a matter of hours. This therefore restricts the major effects of the COX-1 inhibitor to the platelet. It is for these reasons that aspirin is preferred to other non-steroidal antiinflammatory agents in the prophylaxis of thrombotic-based disorders. Treatment with aspirin increases the metabolism of AA via 12-lipoxygenase and 15-lipoxygenase enzymes in platelets, but this appears to have little physiological significance as the metabolites of these two enzymes do not have a major influence on platelet activation.

Recent years have seen rapid developments in our understanding of the regulation of PLA_2 in platelets, and of the pathways that govern the metabolism of the liberated AA. In this chapter, we will describe the major forms of PLA_2 in platelets and their regulation and give only brief details on the metabolism of AA. Comprehensive reviews on the action of TxA_2 and other eicosanoids on platelet function have been published[6,7].

Metabolism of AA in platelets

AA is an essential fatty acid that is stored in the *sn*-2 position of membrane phospholipids. It is the most prevalent fatty acid in platelets, and is found in particularly high levels in phosphatidylcholine, phosphatidylethanolamine

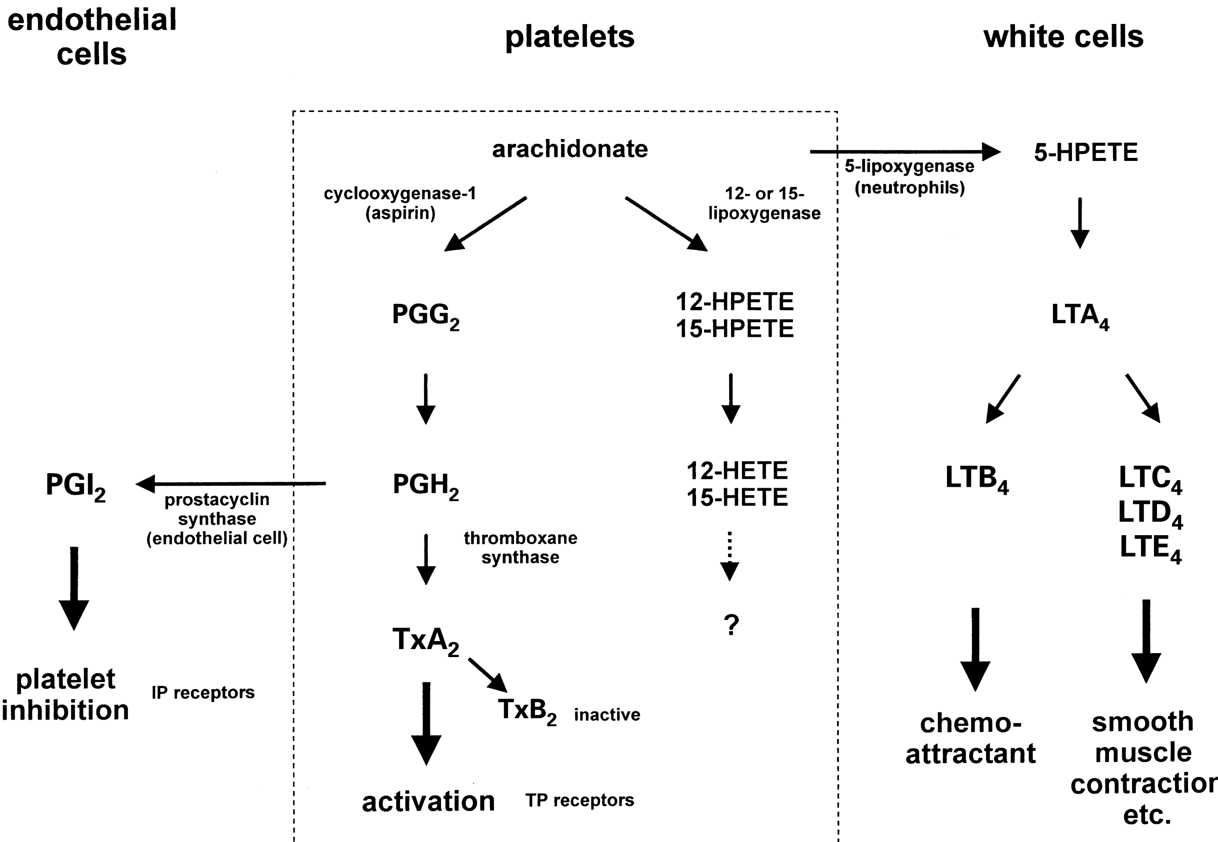


Fig. 15.1. Metabolism of AA in platelets and other cells. The metabolism of AA in platelets is shown in the dotted box. AA is converted by the action of cyclooxgenase-1 to the endoperoxides PGG_2 and PGH_2 and then to thromboxane A_2 (TxA_2), which stimulates platelet aggregation via the thromboxane receptor, TP, which is coupled to Gq-dependent activation of phospholipase C. PGH_2 is also released from the platelet and converted to the inhibitory prostaglandin, prostacyclin (PGI_2), in endothelial cells. PGI_2 inhibits platelet activation via the prostanoid receptor, IP, which is coupled to adenylyl cyclase. AA is also metabolized via 12- and 15-lipoxygenases to 12- or 15-hydroperoxyeicosatetraenoic acid (HPETE) and then to 12- or 15-hydroxyeicosatetraenoic acid. The physiological significance of this is uncertain as the products have no known functional roles in the platelet. The release of AA from platelets is available for conversion to proinflammatory leukotrienes by white cells.

and phosphatidylinositol. Phosphatidylcholine and phosphatidylethanolamine contain over 70% of the total AA in the cell. The AA-containing lipids are localized to the inner leaflet of the plasma membrane and membranes of intracellular organelles, with approximately similar levels in the two compartments. There is evidence that phosphatidylcholine and phosphatidylethanolamine, or possibly just the latter, are the major sources of AA following receptor stimulation (for discussion see[6,7]). Although AA is the predominant fatty acid in the *sn*-2 position of phospholipids, PLA_2s are able to hydrolyse phospholipids with other acyl chains. This is of particular relevance to dietary supplementation with fish oils enriched in ω-3 fatty acids, including eicosapentaenoic acid. The liberated eicosapentaenoic acid is converted to the inactive thromboxane A3, contributing to the antiplatelet effects of fish oil.

The AA that is liberated by the action of PLA_2 is available for metabolism via COX-1 or lipoxygenase pathways. COX-1 is the major AA metabolizing enzyme that is present in the platelet. In addition, platelets express 12-lipoxygenase and, in lower levels, 15-lipoxygenase, but lack the 5-lipoxygenase which is required for generation of leukotrienes (see Fig. 15.1). The major metabolite of AA in platelets is TxA_2. This is generated by the double oxygenation of AA yielding PGG_2 and then PGH_2, which, collectively, are known as the endoperoxides. PGH_2 is converted to TxA_2 by thromboxane synthase. TxA_2 is a powerful platelet agonist, inducing activation via the TP

Cambridge Books Online © Cambridge University Press, 2009

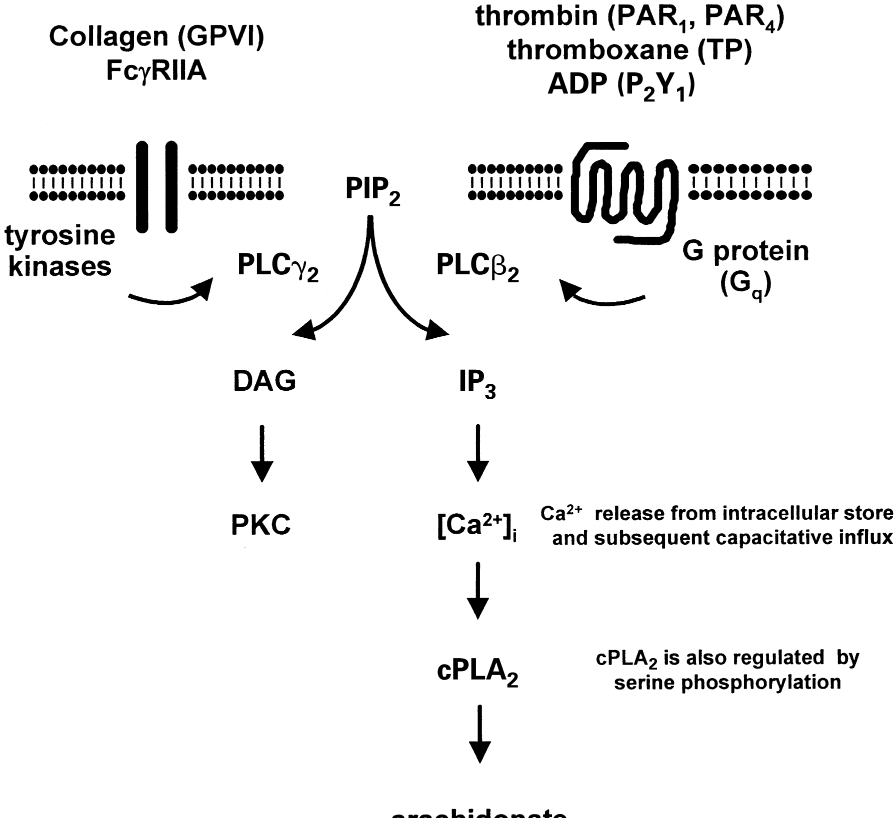


Fig. 15.2. Receptor liberation of AA in platelets. Many platelet agonists mediate activation through stimulation of phospholipase C (PLC) leading to formation of IP_3 and elevation of intracellular Ca^{2+}. These agonists can be subdivided into those which signal via tyrosine kinase-linked and G protein-coupled activation of PLCγ2 and PLCβ2, respectively. The newly generated Ca^{2+} activates $cPLA_2$-α. In addition, $cPLA_2$-α is regulated by phosphorylation on serines 505 and 727 via MAP kinases and MAP kinase-activated protein kinases (see text).

thromboxane receptor, which is coupled to activation of Gq and phospholipase C (Fig. 15.2). Because of the powerful activating effect of TxA_2, and beneficial effects resulting from conversion of released PGH_2 to prostacyclin in endothelial cells (see below), it was thought that inhibitors of thromboxane synthase would be good antiplatelet agents. These inhibitors would have the dual effect of prevention of TxA_2 generation and shunting of PGH_2 to prostacyclin in vascular endothelial cells. It has since been realized, however, that PGH_2 has reasonable activity at the TP receptor[8], making such inhibitors less effective than aspirin.

PGH_2 is also converted in much lower levels to PGD_2, PGE_2 and $PGF_2\alpha$. Platelets do not have specific receptors for PGE_2 and $PGF_2\alpha$, whereas the DP receptor for PGD_2 is present at a low level in some species. In general, the much lower levels of these three prostanoids means that they are of limited physiological significance in platelet function. The newly formed endoperoxides PGG_2/PGH_2 diffuse out of the platelet to the surrounding endothelial cell layer where they are converted to prostacyclin, one of the most powerful inhibitors of platelet activation. Prostacyclin activates IP prostanoid receptors on the platelet surface leading to elevation of cAMP. Physiologically, this helps to prevent progression of the platelet aggregate over the intact endothelium by acting as a physiological antagonist against most platelet agonists. Endothelial cells do not have thromboxane synthase and so cannot generate prostimulatory TxA_2.

The other product produced in reasonable levels in platelets following liberation of AA is 12-hydroxyperoxy-eicosatetraenoic acid (12-HPETE) (Fig. 15.1). Although HPETE has no effect on its own, it has been reported to convert the response of subthreshold concentrations of

Cambridge Books Online © Cambridge University Press, 2009

arachidonic acid to full aggregation at physiological concentrations[9]. This is mediated through potentiation of metabolism of arachidonic acid to thromboxane A_2. At higher, non physiological concentrations, HPETE inhibits platelet responses to arachidonic acid. In addition, HPETE is converted to 12-hydroxyeicosatetraenoic acid (12-HETE) which is released and metabolized to 12,20-HETE in neutrophils, where it may competitively inhibit metabolism of LTB_4[10]. Conversely, platelets convert neutrophil-derived leukotrienes into proinflammatory lipoxins[11]. There is also evidence that AA released from platelets is converted by 5-lipoxygenase to proinflammatory leukotrienes in white cells. The clinical significance of the interaction with neutrophils, however, is uncertain, but emphasizes the potential for cross-talk with other cells.

There is limited evidence to suggest that AA is a second messenger in its own right, although the physiological significance of this remains unclear. For example, AA has been shown to activate protein kinase C in the platelet cytosol independent of Ca^{2+} and phospholipids[12].

Mammalian PLA_2s

There is a diverse collection of PLA_2s in mammals, some of which are secreted whilst others remain intracellular. Secreted PLA_2s ($sPLA_2$) are Ca^{2+}-dependent, disulfide-rich, 14-18 kDa enzymes. The first mammalian $sPLA_2$ to be identified was the group IB enzyme, also known as pancreatic $sPLA_2$. This $sPLA_2$ is found at high levels in pancreatic juice, where it has a well-known function in the digestion of dietary phospholipids[13], but also at lower levels in lung, liver, spleen, kidney, and ovary where it has been proposed to play a role in cell proliferation, acute lung injury, cell migration, and endotoxic shock[14–16]. The second mammalian $sPLA_2$ to be identified was the group IIA enzyme which is expressed at high levels during inflammation[17], and is the principal bactericidal agent against Gram-positive bacteria in human tears[18].

In addition to the above roles, it is becoming clear that $sPLA_2$s are involved in a diverse set of physiological functions[14,19–21]. In the last few years, six mouse and five human $sPLA_2$s structurally related to groups IB and IIA $sPLA_2$s (mouse groups IIC, IID, IIE, IIF, V, and X, and human groups IID, IIE, IIF, V, and X) have been identified[22–27]. All of these group I/II/V/X $sPLA_2$s have similar primary structures, including identical catalytic site residues and partially overlapping sets of disulfides[28]. However, they are not closely related isoforms since the level of amino acid identity is typically 20-50% among these $sPLA_2$s. More recently, a novel human group III $sPLA_2$ was identified[29], which is structurally distinct from the group I/II/V/X $sPLA_2$s but related to the group III $sPLA_2$s found in bee and lizard venom. Very recently, a new human $sPLA_2$, group XII, was identified that is structurally distinct from all previously characterized mammalian and venom $sPLA_2$s and shares homology with known $sPLA_2$s only in short active site segments containing catalytic residues[30]. This diversity of $sPLA_2$ structures and the fact that the tissue distribution of the different $sPLA_2$s are distinct argue for a range of physiological functions for these lipolytic enzymes.

Mammalian cells also contain intracellular PLA_2s. Cytosolic PLA_2 ($cPLA_2$-α) is an 87-kDa protein that binds to membranes in a Ca^{2+}-dependent manner[31]. $cPLA_2$-α contains a C2 domain at its N-terminus which constitutes the Ca^{2+}-dependent membrane binding element[32–34]. Many of the $sPLA_2$s described above have been tested for *sn*-2 fatty acyl chain selectivity and are found to display little, if any, specificity for polyunsaturated versus saturated or monounsaturated chains[35]. In contrast, $cPLA_2$-α displays significant specificity for the *sn*-2 arachidonyl chain over the *sn*-2 oleate chain for example[35]. In response to a rise in intracellular calcium to the low micromolar range, $cPLA_2$-α transfers from the cytosol to the perinuclear region (nuclear membrane and what appears to be nearby membrane elements such as a portion of the Golgi and endoplasmic reticulum)[34,36,37]. The binding of $cPLA_2$-α to the intermediate filament protein vimentin may mediate this process[38]. Studies with $cPLA_2$-α-deficient mice provide strong evidence that $cPLA_2$-α plays a role in AA release for the biosynthesis of eicosanoids in inflammatory cells[39–42]. Two new human $cPLA_2$-α paralogs, $cPLA_2$-β and $cPLA_2$-γ, have been recently cloned based on searching genomic databases[43,44], and no physiological functions have been assigned to these enzymes.

Mammalian cells also contain an intracellular calcium-independent PLA_2 ($iPLA_2$)[45]. Studies using an $iPLA_2$ inhibitor as well as antisense technology suggest that this enzyme is involved in phospholipid remodelling in which the *sn*-2 chain of newly synthesized phospholipids is eventually replaced with a polyunsaturated fatty acyl chain[45]. $iPLA_2$ may not carry out this function in all mammalian cells[46]. $iPLA_2$ may be responsible for AA release seen in some mammalian cells undergoing apoptosis[47]. Multiple $iPLA_2$ splice variants have been identified[48] as well as a human paralog, $iPLA_2$-γ[49], but the functions of these enzymes are not known.

PLA_2s in platelets

At the time of this writing, platelets are known to contain two PLA_2s, group IIA $sPLA_2$ and $cPLA_2$-α. Kramer and

Cambridge Books Online © Cambridge University Press, 2009

coworkers discovered human group IIA $sPLA_2$ by purifying it from platelets[50]. Partial amino acid sequence obtained from the purified enzyme led to isolation of the human group IIA $sPLA_2$ genomic clone. They also showed that human group IIA $sPLA_2$ is secreted in active form following stimulation of platelets with thrombin. Whereas pancreatic-type (group IB) $sPLA_2$ is secreted as an inactive form with a propeptide and requires proteolytic activation, human group IIA does not contain a propeptide, only a signal peptide. Early studies also showed that rat and rabbit platelets contain group IIA $sPLA_2$[51–53]. Intravenous injection of ADP into rats leads to a rapid rise in plasma PLA_2 activity probably coming from activated platelets[54]. This enzyme is known to bind tightly to heparin, and after secretion from platelets may absorb onto heparan sulfate proteoglycan present on the surface of vasculature endothelial cells. Eventually, group IIA $sPLA_2$ in plasma is taken up by the liver and degraded.

$cPLA_2$-α was also purified from human platelets, leading to sufficient material for partial amino acid sequencing and cloning[55]. Bovine and rabbit platelets are also reported to contain $cPLA_2$-α[56,57]. As will be discussed in detail below, this enzyme is activated in agonist-stimulated platelets by phosphorylation and by a rise in intracellular Ca^{2+}.

Role of $cPLA_2$-α in platelet AA release

Two lines of evidence suggest that $cPLA_2$-α is responsible for much of the AA release in platelets following stimulation with the physiological agonists thrombin and collagen. The first comes from the use of a $cPLA_2$-α-specific inhibitor $AACOCF_3$[58,59]. This compound is an AA analogue in which the COOH group is replaced with a trifluoromethyl ketone group $COCF_3$. It is thought that $AACOCF_3$ forms a stable hemiketal adduct with the active site serine of $cPLA_2$-α (Ser-228) that resembles the tetrahedral intermediate that forms from the attack of this serine onto the carbonyl carbon of the substrate ester undergoing hydrolysis. $AACOCF_3$ does not inhibit $sPLA_2$s, although it does inhibit $iPLA_2$[60]. Two independent studies show that low micromolar concentrations of $AACOCF_3$ block virtually all of the AA released in human platelets stimulated with thrombin and calcium ionophore[61,62]. The control compounds $AACOCH_3$ and $AACH(OH)CF_3$, which do not inhibit $cPLA_2$-α in vitro, fail to block AA release in platelets. $AACOCF_3$ was further reported to block downstream oxygenation of AA in platelets[62], suggesting it may also act as a competitive inhibitor of oxygenating enzyme COX-1.

The second line of evidence that $cPLA_2$-α is involved in platelet AA release comes from studies of the regulation of this enzyme (described in detail below). Studies in human platelets show that $cPLA_2$-α becomes phosphorylated and activated in platelets stimulated with a variety of agonists[63,64]. This post-translational modification occurs with the same time course as AA release in platelets. $cPLA_2$-α is also phosphorylated and activated in a variety of other mammalian cells[31,65]. Although $cPLA_2$-α-deficient mice are available[39,40], studies of AA release in $cPLA_2$-α-deficient platelets have not been reported.

Regulation of $cPLA_2$-α in platelets

It has been proposed that AA liberation in platelets may be regulated by GTP-binding protein(s) and protein kinase C. Such studies were performed with platelet-membrane fractions or permeabilized platelets, and, platelet activation was performed with non physiological agonists such as PMA or fluoroaluminate[66–69]. By contrast, studies on the regulation of $cPLA_2$-α by phosphorylation and by Ca^{2+} have been reported in intact platelets activated by physiological agonists such as thrombin and collagen. Regulation of $cPLA_2$-α by phosphorylation and Ca^{2+} has also been studied in several other mammalian cells[31]. In this part, we will mainly focus our review on the regulation of $cPLA_2$-α that occurs during platelet stimulation by thrombin and collagen.

Regulation of platelet $cPLA_2$-α by Ca^{2+}

Because of the pivotal role of intracellular Ca^{2+} in the regulation of $cPLA_2$-α via its C2 domain, it follows that agonists that increase the levels of the cation also bring about the activation of $cPLA_2$-α. For the majority of agonists, the increase in Ca^{2+} is mediated through IP_3-dependent release of Ca^{2+} from intracellular stores and capacitative Ca^{2+} entry. The increase in IP_3 is brought about by G protein-coupled surface receptors, which activate phospholipase $C\beta_2$ ($PLC\beta_2$) isoforms, and tyrosine kinase-linked receptors, which activate $PLC\gamma_2$ (Fig. 15.2). Examples of G protein-coupled receptors that activate $PLC\beta_2$ downstream of Gq include the thrombin receptors, PAR_1 and PAR_4, the ADP receptor, P_2Y_1, and the thromboxane receptor, TP. Additionally, it has been proposed that the liberation of G protein $\beta\gamma$ subunits by Gi-coupled receptors such as the ADP receptor, P_2Y_1, P_2Y_2 and a_2-adrenoceptor, potentiates activation of $PLC\beta_2$ by Gq, although direct evidence for this is lacking. The major tyrosine kinase-linked receptors in human platelets are the collagen receptor GPVI and the platelet low affinity immune receptor, $Fc\gamma$RIIA. The depletion of intracellular Ca^{2+} stores by IP_3 leads to influx of Ca^{2+} by capacitative entry

Cambridge Books Online © Cambridge University Press, 2009

and a correspondingly greater increase in AA release. There is also emerging evidence for additional pathways of Ca^{2+} entry that are independent of IP_3 such as direct regulation of cation channels by tyrosine kinases[70]. There have been no studies describing the site of translocation of cPLA$_2$-α in the platelet, although it is noteworthy that studies in other cells have reported translocation to intracellular membranes[34,36,37]. It is of particular interest to investigate whether cPLA$_2$-α translocates to dense tubular membranes, the site of location of COX-1 and thromboxane synthase in platelets[71].

There is limited evidence that cPLA$_2$-α is regulated by receptors in platelets which do not elevate intracellular Ca^{2+}. The cytokine thrombopoietin potentiates platelet activation by a wide range of agonists, but has no stimulatory action on its own. Phosphorylation of cPLA$_2$-α has been proposed as the mechanism underlying the priming action of thrombopoietin[72]. This is not the only mechanism of priming, however, as thrombopoietin potentiates activation in the presence of cyclooxygenase inhibitors[73]. The physiological significance of the priming effect of the cytokine is unclear. There is limited evidence that the major platelet integrin, αIIb-β3, which is a receptor for fibrinogen and von Willebrand factor, can activate cPLA$_2$-α.

Regulation of cPLA$_2$-α by phosphorylation

Lin and coworkers at Genetic Institute in Boston were the first to provide strong evidence that cPLA$_2$-α is phosphorylated in mammalian cells in response to agonists that mobilize AA from phospholipids[74]. After this group cloned cPLA$_2$-α, they realized that the protein contains a consensus motif surrounding Ser-505 for phosphorylation by mitogen activated protein kinase family members (MAPKs). This group also provided strong evidence, but not proof, that cPLA$_2$-α is phosphorylated on Ser-505 when expressed in CHO cells. When the S505A cPLA$_2$-α mutant was expressed in CHO cells, AA release in response to Ca^{2+} ionophore and the protein kinase C activator PMA was greatly decreased compared to that produced by expression of wild-type cPLA$_2$-α. They also showed that cPLA$_2$-α phosphorylation led to a slight decrease in the electrophoretic mobility of the enzyme. This gel shift assay, which can be examined by immunoblotting methods using total protein extracted from mammalian cells, has been used by many investigators to track cPLA$_2$-α phosphorylation in response to agonists (> 300 published studies).

However, we were never completely satisfied with the gel shift assay because it does not provide site-selective cPLA$_2$-α phosphorylation data. We were able to isolate sufficient quantities of cPLA$_2$-α from a baculovirus/insect cell expression system to determine the sites of phosphorylation by protein chemical methods. We determined that cPLA$_2$-α was phosphorylated not only on Ser-505 but also on serines-437, 454, and 727[75]. This was accomplished by using combined HPLC/electrospray mass spectrometry to fully sequence the four tryptic phosphopeptides. Radiometric methods with ^{32}P were used to show that these four phosphorylations accounted for all of the cPLA$_2$-α phosphorylation in these cells. We then studied cPLA$_2$-α phosphorylation in human platelets following stimulation with the physiological agonists thrombin and collagen. Although sufficient amounts of cPLA$_2$-α from mammalian cells for mass spectrometry studies cannot be readily obtained, we were able to use ^{32}P-radiometric methods to show that the radiolabelled tryptic peptides obtained from immunoprecipitated platelet cPLA$_2$-α comigrated with authentic phosphopeptides (prepared by solid-phase peptide synthesis) on both a reverse-phase HPLC column and on a two-dimensional cellulose plate (electrophoresis followed by thin layer chromatography). By this method, we showed that cPLA$_2$-α is phosphorylated in thrombin and collagen stimulated platelets on Ser-505 and on Ser-727, and this accounted for all of the phosphorylation in these cells (Ser-437 and Ser-454 phosphorylations were not observed)[76] (Fig. 15.3). Since cPLA$_2$-α phosphorylation is stoichiometric in platelets (full gel-shift) and since the cpm incorporated at Ser-505 and Ser-727 are virtually identical, we conclude that platelet cPLA$_2$-α becomes stoichiometrically phosphorylated on both serines in response to thrombin. We also showed that cPLA$_2$-α is phosphorylated exclusively on Ser-505 and on Ser-727 in HeLa cells stimulated with activators of the stress-activated protein kinases (SAPKs, members of the MAPK family)[76]. These are the only published studies reporting the rigorous mapping of cPLA$_2$-α phosphorylation sites. Our work cast doubt on the suggestion, based only on immunoblot analysis, that cPLA$_2$-α is phosphorylated on tyrosine in HeLa cells[77].

We, along with Kramer and coworkers, showed that the p38 member of the SAPK family or a closely related kinase and not the p42/p44 MAPKs (ERK1 and 2) was responsible for cPLA$_2$-α phosphorylation in platelets[78–81]. We found that the inhibitors of p38, SB202190 and SB203580, blocked about 50% of the phosphorylation on both Ser-505 and Ser-727 in thrombin stimulated platelets. Since the Ser-727 site does not contain the critical residues for recognition by MAPKs, the inhibition data suggests that the kinase responsible for Ser-727 phosphorylation is activated by p38. The fact that cPLA$_2$-α phosphorylation is only 50% inhibited by SAPK inhibitors suggests that more than one isoform of p38 (inhibitor sensitive and insensitive

Cambridge Books Online © Cambridge University Press, 2009

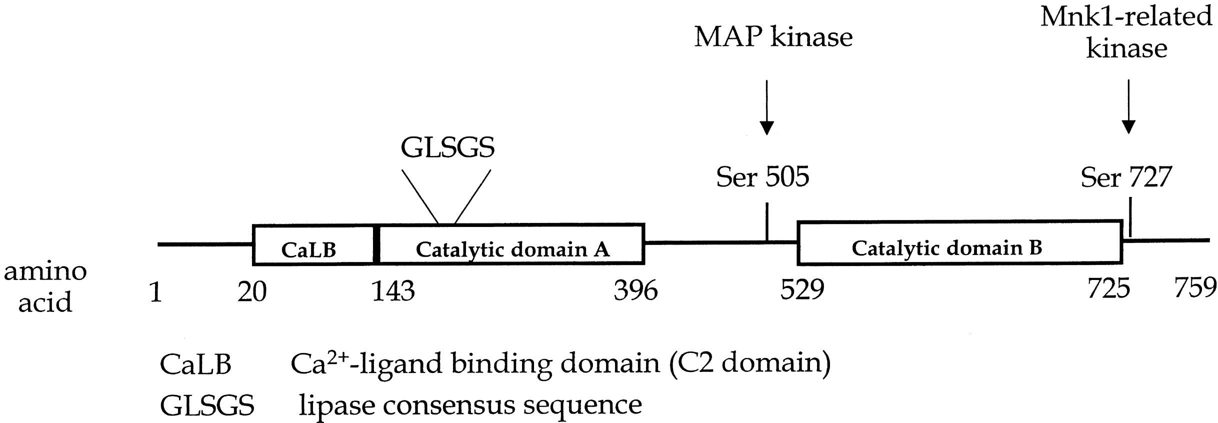


Fig. 15.3. Schematic representation of major structural domains of the cPLA$_2$-α. The catalytic domain composed of two parts of the protein (catalytic domain A and catalytic domain B), the Ca^{2+}-binding domain are indicated as well as two phosphorylation sites.

forms) may be phosphorylating cPLA$_2$-α in platelets. Since these studies, three new p38 isoforms have been cloned[82–85], bringing the current count to four gene products (SAPK2a/2b/3/4). Using recombinant SAPKs and specific antibodies, we went on to show that all four SAPKs phosphorylate cPLA$_2$-α in vitro at Ser-505[78]. All four SAPKs are present in platelets and SAPK2a/2b and four undergo activation in thrombin-stimulated platelets[78]. However, SB202190 and SB203580 inhibit SAPK2a/2b but not SAPK3/4[86]. Thus, it seems that the partial inhibition of cPLA$_2$-α phosphorylation by the SB202190/SB203580 inhibitors may be due to inhibitor sensitive and insensitive forms of SAPKs that phosphorylate cPLA$_2$-α upon thrombin addition. Alternatively, the SB202190/SB203580-insensitive phosphorylation could be mediated by other members of the MAPK family such as ERK5 or Jnk. In collagen stimulated platelets, only the SB202190/SB203580-sensitive SAPKs, SAPK2a/$_2$b, become activated, and we found that these inhibitors block all of the cPLA$_2$-α phosphorylation (Ser-505 and Ser-727) induced by collagen[78]. We then went on to show that complete blockage of cPLA$_2$-α phosphorylation leads to a shift in the AA release vs. collagen concentration dose–response curve in platelets. Threefold higher amounts of collagen are needed to elicit the same AA release response in the absence of cPLA$_2$-α phosphorylation (i.e. with SB202190/SB203580 present) as in the presence of phosphorylation[78]. We also showed that stress stimuli that activate platelet SAPKs (oxidants and osmotic shock) lead to enhanced cPLA$_2$-α phosphorylation and AA release in platelets[87], consistent with a role of SAPK-catalyzed cPLA$_2$-α phosphorylation in augmenting AA release in platelets.

Kramer et al. showed that phorbol ester activation of the p42/p44 MAPK members in platelets leads to cPLA$_2$-α phosphorylation, and we showed that such phosphorylation also occurs on Ser-505 and Ser-727[65]. It should be noted that this cPLA$_2$-α phosphorylation pathway is an induced response to the non-physiological phorbol ester agonist; as noted above, cPLA$_2$-α phosphorylation is catalysed by SAPKs in response to the physiological agonists thrombin and collagen.

Our studies with SAPK inhibitors SB202190/SB203580 led us to propose that the kinase responsible for phosphorylation of cPLA$_2$-α on Ser-727 is activated by one or more SAPKs. The identification of MAPK-activated kinases is rapidly advancing, and we decided to test all of the kinases known at the time of our study to be activated by both p42/p44 MAPKs and by SAPKs (MNK1[88,89], PRAK1[90], MSK1[91], MAPKAP-K2[89], and MAPKAP-K3[92]) for their ability to phosphorylate cPLA$_2$-α in vitro. MAPKAP-K1 ($p90^{rsk}$) is not activated by SAPK2a, but lies downstream of ERK2[86,93] and thus was not considered further. Using mass spectrometry, we proved that MNK1 phosphorylates cPLA$_2$-α in vitro uniquely at Ser-727 and that PRAK1 and MSK1 produce a radiophosphorylated tryptic peptide that co-migrates with authentic Ser-727 phosphorylated tryptic peptide on HPLC and on a two-dimensional cellulose plate[65]. MAPKAP-K2 and MAPKAP-K3, although enzymatically active, did not phosphorylate cPLA$_2$-α in vitro. Using specific antibodies, we showed that MNK1 and PRAK1, but not MSK1, are present in platelets and become activated upon thrombin addition and with the same time course as activation of SAPKs and cPLA$_2$-α phosphorylation[65]. At this point it seems clear that cPLA$_2$-α phosphorylation on Ser-727 in activated platelets is carried out by MNK1, PRAK1, or a closely related isoform.

Cambridge Books Online © Cambridge University Press, 2009

Having defined that pattern of $cPLA_2$-α phosphorylation in platelets and the family of kinases responsible for the modifications, we further examined the functional consequence of Ser-505 and Ser-727 $cPLA_2$-α phosphorylation[65]. We constructed a mammalian cell expression plasmid containing mouse $cPLA_2$-α cDNA and prepared several clones of stably transfected CHO cells, HeLa cells, and HEK293 cells (human embryonic kidney endothelial cells). Clones were also established that expressed the phosphorylation site mutants (S505A, S727A, and S505A/S727A). We studied $cPLA_2$-α phosphorylation in these cells by radiolabelling with ^{32}P-phosphate and examining the radioactive tryptic peptides derived from immunoprecipitated $cPLA_2$-α by HPLC and 2-dimensional cellulose chromatography. We found that $cPLA_2$-α was phosphorylated stoichiometrically only on Ser-505 and Ser-727 in all three cell types and that mutation of these serines to alanines abolished phosphorylation, thus confirming our phosphorylation site mapping studies.

We next quantified AA release in these clones in response to agonist stimulation[65]. Several clones expressing each type of $cPLA_2$-α were selected for study based on comparable amounts of expressed $cPLA_2$-α (based on immuno- and RNA-blots)[65]. Similar results were obtained with CHO, HeLa, and HEK293 cells[65]. Expression of wild-type $cPLA_2$-α in CHO cells leads to an increase in AA release compared to the non-transfected parental cells. Stimulation with an intermediate concentration of Ca^{2+} ionophore alone leads to more AA release than stimulation with the protein kinase C agonist PMA alone, and there is a synergistic effect when both agonists are added. These results are similar to those reported by Lin et al. for $cPLA_2$-transfected CHO cells[74]. Mutation of either Ser-505 or Ser-727 of $cPLA_2$-α significantly reduces AA release, and the double mutant fails to produce AA above the level made by non-transfected cells. When a high concentration of Ca^{2+} ionophore is used, the effect of double mutation on AA release is rescued showing that the mutant can be fully functional in these cells. These results establish that phosphorylation of $cPLA_2$-α on Ser-505 and on Ser-727 are important for efficient AA release and that high Ca^{2+} concentrations can overcome the need for $cPLA_2$-α phosphorylation. Finally, we prepared HEK293 cell double transfectants that overexpress wild-type $cPLA_2$-α and a dominant negative form of MNK1[94]. Expression of dominant negative MNK1 led to a significant reduction in AA release, but when HEK293 cells were stimulated with high Ca^{2+} ionophore concentration, no effect of dominant negative MNK1 was observed[65]. These results support our earlier studies that $cPLA_2$-α is phosphorylated by MNK1 or a closely related kinase in platelets and that phosphorylation of $cPLA_2$-α is not required for AA release in the presence of high intracellular Ca^{2+}. Our studies also underscore the need for rigorous analysis of $cPLA_2$-α phosphorylation using site-selective protein chemical techniques rather than simply relying on gel shift analysis.

In the early studies by Kramer et al. it was shown that fully phosphorylated $cPLA_2$-α partially purified from thrombin-stimulated human platelets is about two- to threefold more active than non-phosphorylated enzyme toward the hydrolysis of phospholipid vesicles in vitro[95]. Phosphorylation is unable to bring about activation of $cPLA_2$-α on its own, but increases the response to elevation of Ca^{2+}. This can be explained by the ability of Ca^{2+} to stimulate movement of $cPLA_2$-α to intracellular membranes, whereas phosphorylation increases the catalytic efficiency of the membrane-bound enzyme. In support of this, mutational studies have shown that phosphorylation of serine-505 does not play a role in translocation to intracellular membranes[37]. Thus, in combination, Ca^{2+} and phosphorylation induces a larger increase in activity than either stimulus on its own.

Evidence that group IIA $sPLA_2$ may not be involved in AA release in platelets stimulated with physiological agonists

Group IIA $sPLA_2$ has been detected in α-granules of platelets from different species including rat, rabbit and human and is secreted within a few minutes upon activation by physiological agonists[50,96,97]. The question of the possible involvement of group IIA $sPLA_2$ in AA liberation during platelet activation has been investigated by two different approaches. First, addition of a specific inhibitor for group IIA $sPLA_2$ did not inhibit AA liberation from thrombin-stimulated platelets, while specific inhibition of $cPLA_2$-α led to a strong decrease in such a liberation[61]. Secondly, it was shown that group IIA $sPLA_2$-depleted platelets aggregated as much as control platelets and produced similar amounts of TxA_2 upon agonist stimulation[97]. These data lead to the conclusion that group IIA $sPLA_2$ does not play a significant role in AA liberation upon agonist stimulation. Natural strains of mice lack group IIA $sPLA_2$[98], and it would be interesting to examine AA and TxA_2 release in platelets derived from these strains.

Since thrombosis and inflammation are two closely related processes, it is possible that once released from platelets, group IIA $sPLA_2$ could participate in eicosanoid and/or biologically active lysophospholipid formation in other inflammatory cells or from microvesicles and therefore lead to the propagation of the inflammatory

Cambridge Books Online © Cambridge University Press, 2009

process[99,100]. The addition of group IIA $sPLA_2$ on A23187-treated granulocytes, IgE/antigen-primed mast cells, TNFα-stimulated HUVEC and BRL-3A cells, leads to more prostanoids generated than that elicited by each agonist alone[101–104]. Moreover, group IIA $sPLA_2$ does contribute to AA release for eicosanoid formation in epithelial and fibroblastic cells[20]. In these cells, group IIA $sPLA_2$ seems to contribute to AA release during the late phase of eicosanoid formation, typically tens of minutes to hours after cell activation, whereas $cPLA_2$-α contributes to the early phase of AA release. In this context, it is important to note that platelets release AA and TxA_2 within a few minutes after stimulation by physiological agonists. Once secreted from platelets, group IIA $sPLA_2$ is expected to adsorb to the plasma membrane of nearby cells, rather than being diluted into the systemic circulation. This is due to its ability to bind heparan sulfate proteoglycan present at the cellular surface[20,105,106]. In fibroblastic cells, group IIA $sPLA_2$ anchored to cell surface heparan sulfate proteoglycan is internalized in a process that leads to AA release coupled to eicosanoid formation[106]. In contrast, with mast cells, such anchoring leads to group IIA $sPLA_2$ internalization for degradation[107].

The ability of exogenous added group IIA $sPLA_2$ to release AA from platelets was also studied. Addition of micromolar concentrations of exogenous enzyme to resting platelets does not induce platelet aggregation, AA liberation, or plasma membrane phospholipid hydrolysis[61,108,109]. It therefore appears that the extracellular face of the platelet plasma membrane is a poor substrate for group IIA $sPLA_2$. This is probably due to the fact that this membrane face is highly enriched in sphingomyelin and phosphatidylcholine[110,111], and that group IIA $sPLA_2$ binds extremely weakly to vesicles of zwitterionic phospholipids[112]. Phosphatidylserine is known to be transferred to the extracellular face of the plasma membranes of platelets during cell activation. However, this change in membrane composition is not sufficient to allow group IIA $sPLA_2$ to act since little or no AA is released when activated platelets are treated with exogenous addition of group IIA PLA_2[61,109]. Addition of phosphatidylserine to phosphatidylcholine vesicles leads to enhanced binding of group IIA $sPLA_2$ in vitro, but interfacial binding of enzyme remains weak unless the concentration of acidic phospholipid lipid approaches 100 mole per cent (M.H. Gelb, unpublished observations).

One possible hydrolytic action of the group IIA $sPLA_2$ is on microvesicles shed from platelets during activation by thrombin and collagen[108,113]. Such microvesicles are enriched in phosphatidylserine and phosphatidylethanolamine in the outer leaflet. Micromolar concentrations of group IIA $sPLA_2$ induced a modest phospholipid hydrolysis on such microvesicles after one-hour incubation, while a marked hydrolysis was observed in the presence of sphingomyelinase[108]. The physiological relevance of such a process remains to be established, but it is unlikely it would contribute significantly to AA liberated from platelets during thrombin or collagen stimulation. The shedding of microvesicles is a slow process, whereas AA release is complete within a few minutes[114,115]. In addition, weak agonists which are unable to induce microvesicle shedding are potent inducers of eicosanoid formation during platelet activation[115,116]. However, the possibility that physiologically relevant signaling lipids including lysophosphatidic acid and lysophosphatidylcholine are produced during group IIA $sPLA_2$-catalysed microvesicle hydrolysis needs to be considered.

In conclusion, available evidence goes against a role for group IIA $sPLA_2$ in generating AA during physiological agonist stimulation of platelets. In light of the fact that several new $sPLA_2$s have been discovered in recent years (discussed above), it will be important to explore the presence of these enzymes in platelets. It should also be mentioned that the action of an $sPLA_2$ other than the group IIA enzyme could contribute to AA release during platelet activation. It has been shown that the action of $cPLA_2$-α is needed as a prerequisite for $sPLA_2$ action in mast cells[117] and in fibroblasts[118]. This complicates the interpretation of the results that $AACOCF_3$ blocks virtually all of the AA released in activated platelets, but the results with this inhibitor show that $cPLA_2$-α plays the dominant role in liberating AA from platelet membrane phospholipids for the biosynthesis of eicosanoids.

Possible physiological functions of platelet-derived group IIA $sPLA_2$

While the synthesis and the subsequent secretion of group IIA $sPLA_2$ via the classical secretory pathway in many cell-types is regulated by proinflammatory stimuli[20,119,120] (Il-1, Il-6, TNF, LPS), in some cells group IIA $sPLA_2$ is rapidly released from storage granules following cell activation. For example, group IIA $sPLA_2$ is stored in α-granules of resting platelets and released along with other granule proteins during activation. Such a secretion is correlated with the activation state of blood platelets and with the efficiency of the release reaction, but specific regulation of group IIA $sPLA_2$ secretion compared to other granule proteins has not been reported. It is likely that platelet-derived group IIA $sPLA_2$ mainly exerts its physiological functions once secreted since no report of an action of group IIA

Cambridge Books Online © Cambridge University Press, 2009

Table 15.1. The possible biological functions for platelet-derived group IIA $sPLA_2$

Reported properties for group IIA $sPLA_2$	Expected biological functions for platelet-derived group IIA $sPLA_2$
Binding to heparan sulfate proteoglycans	Extracellular or intracellular participation in AA production by cells other than platelets
Intracellular degradation of bacteria, extracellular hydrolysis of bacterial phospholipids	Antimicrobial effect
Binding to blood coagulation factor Xa	Anticoagulant effect
Platelet-derived microvesicle hydrolysis	Production of biologically active lipids (LPA, LPC), removal of microvesicles
LDL hydrolysis	Proatherogenic effect
Membrane hydrolysis of cells with a loss in the membrane asymmetry	Removal of apoptotic/injured/activated cells

$sPLA_2$ inside platelets has been described. Group IIA $sPLA_2$ binds tightly to heparin[20,106]. It is possible that it forms a complex with heparin inside of granules which prevents it from acting on granular membranes, but this idea remains to be investigated. It is still a question whether platelet-derived group IIA $sPLA_2$ may account for the high increase in the group IIA $sPLA_2$ level found in serum during inflammatory diseases. In the rat, in vivo platelet activation by administration of ADP was followed by a transient rise in plasma $sPLA_2$ activity[54]. However, it has been reported that other cell-types (such as inflammatory cells and liver cells) also secrete high amount of group IIA $sPLA_2$[20,121]. Group IIA $sPLA_2$ is expected to stay, at least partly, at the cellular surface after secretion (due to its proteoglycan binding properties) rather than being diluted into the systemic circulation[105,106]. High concentrations of group IIA $sPLA_2$ have been detected in local sites, such as synovial[122] and ascitic fluids[123] and in tears[18], and localization of group IIA $sPLA_2$ appears to occur during local thrombolytic events[20,108]. The possible physiological functions of the group IIA $sPLA_2$ will be reviewed mainly with the working hypothesis that it is acting locally and extracellularly after secretion from platelets (Table 15.1). However, an intracellular action of the group IIA $sPLA_2$ after binding to the cell surface and subsequent internalization has been proposed in some cells and discussed above regarding the participation of platelet-derived group IIA $sPLA_2$ in eicosanoid formation by other cells[106]. Finally, it has to be considered that group IIA $sPLA_2$ may also be secreted by other cells present at the thrombus foci and therefore act with the platelet-derived enzyme.

Anticoagulant effect of group IIA $sPLA_2$

The group IIA $sPLA_2$ has been reported to exert an anticoagulant effect and may play a negative feedback role during the initiation of thrombosis once secreted from activated platelets[124,125]. Group IIA $sPLA_2$ specifically inhibits the prothrombinase complex composed of factor Xa (FXa), factor Va (FVa), phospholipids and calcium, and this inhibitory effect is still observed in the absence of phospholipids[125,126]. Group IIA $sPLA_2$ forms a 1:1 complex with FXa and prevents the formation of the FXa/FVa complex[125]. Group IIA $sPLA_2$ contains 13 lysine and 10 arginine residues scattered over its entire surface that form cationic clusters. Several of these basic clusters lie on the surface of the molecule that contacts the lipid membrane, the so-called 'interfacial binding surface'[127]. Mutations of these basic residues led to a significant reduction of the ability of group IIA $sPLA_2$ to inhibit prothrombinase activity and to bind to FXa, and, increased salt concentrations completely abolished the binding of group IIA $sPLA_2$ to FXa, supporting an important role of electrostatic interactions[128]. High concentrations of FVa reversed the inhibitory effect of group IIA $sPLA_2$ on the prothrombinase complex, suggesting that group IIA $sPLA_2$ may compete with FVa for binding to FXa. Therefore, group IIA $sPLA_2$ is expected to act at early stages of the coagulation process by delaying the formation of a fully active prothrombinase complex, until enough FVa is generated.

Wild-type enzyme, as well as the catalytically inactive H48Q group IIA $sPLA_2$, efficiently delays the formation of thrombin measured in human platelet-rich plasma (PRP). Therefore, the anticoagulant effect of group IIA $sPLA_2$ occurs under experimental conditions close to the physiological situation and is independent of its catalytic activity,

Cambridge Books Online © Cambridge University Press, 2009

even when natural phospholipids from plasma and platelet membrane are present, supporting an inhibitory mechanism based on the interaction of group IIA $sPLA_2$ with FXa[128]. As discussed above, the levels of group IIA $sPLA_2$ in serum strongly increase from 0.35 nM up to 600 nM under various inflammatory states[121,129], and, high concentrations are expected to be found at local inflammatory/thrombolytic sites. Group IIA $sPLA_2$ inhibits the prothrombinase activity with an IC_{50} value of 80 nM, and a K_d value of 230 nM was determined for the interaction of group IIA $sPLA_2$ with FXa[128]. These values appear consistent with a potential localized and/or systemic anticoagulant role of group IIA $sPLA_2$ during various physiological or pathological states. Hydrolysis of platelet-derived microvesicles by group IIA $sPLA_2$ (described above) could lead to an anticoagulant effect since these microvesicles support the coagulation cascade in addition to the platelet membrane[113]. However, the catalytically inactive mutant showed the same efficiency as wild-type group IIA $sPLA_2$ to inhibit thrombin generation in PRP, indicating that such phospholipid hydrolysis probably does not play a major role in the anticoagulant effect of this enzyme. Finally, group IIA $sPLA_2$ has been shown to increase the generation of prostacyclin by vascular endothelial cells[103]. This eicosanoid is an efficient inhibitor of platelet activation. The occurrence of such an effect in vivo would amplify the anticoagulant effect of group IIA $sPLA_2$.

Degradation of apoptotic and injured cells by group IIA $sPLA_2$

A membrane rearrangement that could lead to increased hydrolysis by the group IIA $sPLA_2$ is the appearance of phosphatidylserine during the apoptotic process in eukaryotic cells that is correlated with the loss of the membrane asymmetry and with membrane blebbing[130]. Cells undergoing apoptosis are sensitive to the action of the group IIA $sPLA_2$[131,132]. Therefore, one possible function of group IIA $sPLA_2$ released from either platelets or other cells is to eliminate apoptotic cells or injured cells[133]. This may constitute an important role in the removal of the hemostatic clot including activated platelets and microvesicles in order to avoid establishment of a prothrombotic situation.

Role of group IIA $sPLA_2$ in atherogenesis: low density lipoprotein (LDL) hydrolysis

Atherosclerosis is a disease involving the immune system and chronic inflammation in the initiation of endothelial cell dysfunction and the development of advanced lesions[134]. In addition to endothelial cells, macrophages, T-lymphocytes and smooth muscle cells collaborate to induce such a pathological situation. The 'CD40 receptor/CD40 ligand' system, a key mediator of cell communication in the immune system, is crucial in the establishment of atherosclerotic lesions[134]. The possible involvement of blood platelets in such a process has been pointed out since the CD40 ligand is expressed on their surface upon activation, and oxidatively modified LDL (low density lipoprotein) stimulates platelets[135]. The participation of the group IIA $sPLA_2$ in atherogenesis has been recently examined since it is expressed by cells implied in this process[136,137] and detected in human atherosclerotic plaques[138]. It has been shown that group IIA $sPLA_2$ is able to hydrolyse plasma lipoproteins and in particular mildly oxidized or aged LDL leading to modified LDL with higher affinity for proteoglycans and therefore higher atherogenic properties[139,140]. The group IIA $sPLA_2$ binds to heparan sulfate proteoglycans via their glycosaminoglycan moiety and to decorin, a small proteoglycan of the collagen network in atherosclerotic plaques, via the protein core[141]. Indeed, both LDL and group IIA $sPLA_2$ are likely to be colocalized at inflammatory loci via the binding to proteoglycans present on cell membranes and on the extracellular matrix of the arterial wall. Transgenic mice overexpressing group IIA $sPLA_2$ show increased atherogenesis and altered lipoproteins, supporting a role of this enzyme in atherosclerosis[142]. However, the lipoprotein-associated PLA_2 (Lp–PLA_2) which is found predominantly on LDL is also important for the modification of the LDL[143]. The exact function of Lp–PLA_2 vs. group IIA $sPLA_2$ in the establishment of atherosclerosis has still to be further investigated.

Antimicrobial effect of group IIA $sPLA_2$

It has been established that group IIA $sPLA_2$ contributes to the antimicrobial action in response to invading microorganisms[20,144,145]. Phagocytosis and consecutive bacterial membrane degradation by polymorphonuclear leukocytes represent an essential first-line defence against invading bacteria. Group IIA $sPLA_2$ participates in intracellular bacterial digestion by associating with the surfaces of bacteria and neutrophils before phagocytosis and acting after cointernalization with ingested bacteria. Group IIA $sPLA_2$ is also able to efficiently bind and extracellularly hydrolyse the membrane of gram positive bacteria[144]. In order to achieve such phospholipid hydrolysis from gram negative bacteria, group IIA $sPLA_2$ requires the presence of the BPI (bactericidal/permeability-increasing protein) that is released by polymorphonuclear leukocytes[146]. Transgenic mice with the group IIA $sPLA_2$ gene were reported to be more resistant to *Staphyloccocus aureus* and *Escherichia*

Cambridge Books Online © Cambridge University Press, 2009

coli infection than control mice[120,147]. This antimicrobial action is likely to take place in the intestine where group IIA $sPLA_2$ is highly expressed in Paneth cells[148] and in tears[18]. During skin injury often associated with inflammation and thrombosis, it is possible that group IIA $sPLA_2$ secreted either from platelets or inflammatory cells also contribute to eliminate invasive bacteria.

References

1 Collaboration AT. Secondary prevention of vascular disease by prolonged antiplatelet treatment. *Br Med J* 1988; 296: 320–331.
2 Patrono C. Aspirin as an antiplatelet drug. *N Engl J Med* 1994; 330: 1287–1294.
3 Catella-Lawson F, Fitzgerald GA. Therapeutic aspects of platelet pharmacology. *Hanb Exp Pharmacol* 1997; 126: 719–736.
4 Collaboration AT. Collaborative overview of randomised trials of antiplatelet therapy-I: prevention of death, myocardial infarction and stroke by prolonged antiplatelet therapy in various categories of patients. *Br Med J* 1994; 308:81–106.
5 Gent M, Beaumont D, Blanchard J et al. A randomized, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). *Lancet* 1996; 348: 1329–1339.
6 Halushka PV, Pawate S, Martin ML. Thromboxane A_2 and other eicosanoids. *Hanb Exp Pharmacol* 1997; 126: 719–736.
7 Arita H, Nakano T, Hanasaki K. Thromboxane A_2: its generation and role in platelet activation. *Prog Lipid Res* 1989; 28: 273–301.
8 Heptinstall S, Bevan J, Cockbill SR, Parry MJ. Effects of a selective inhibitor of thromboxane synthetase on human blood platelet behaviour. *Thromb Res* 1980; 20: 219–230.
9 Calzada C, Vericel E, Lagarde M. Low concentrations of lipid hydroperoxides prime human platelet aggregation specifically via cyclo-oxygenase activation. *Biochem J* 1997; 325: 495–500.
10 Marcus AJ, Safier LB, Ullman HL et al. Platelet-neutrophil interactions (12S)-hydroxyeicosatetraen-1,20-dioic acid: a new eicosanoid synthesized by unstimulated neutrophils from (12S)dihydroxyeicosatetraenoic acid. *J Biol Chem* 1988; 263: 2223–2229.
11 Serhan SN, Sheppard KA. Lipoxin formation during human neutrophil: platelet interactions. Evidence for transformation of leukotriene A4 by platelet 12-lipoxygenase in vitro. *J Clin Invest* 1990; 85: 772–780.
12 Khan WA, Blobe GC, Hannun YA. Activation of protein kinase C by oleic acid. *J Biol Chem* 1992; 267: 3605–3612.
13 Verheij HM, Slotboom AJ, De Haas GH. Pancreatic phospholipase A_2: a model for membrane-bound enzymes? *Rev Physiol Biochem Pharmacol* 1981; 91: 91–203.
14 Hanasaki K, Arita H. Biological and pathological functions of phospholipase A_2 receptor. *Arch Biochem Biophys* 1999; 372: 215–223.
15 Rae D, Beechey-Newman N, Burditt L, Sumar N, Hermon-Taylor. Activation of human granulocyte type1-prophospholipase A_2. *Scand. J. Gastroenterol Suppl* 1996; 219: 24–27.
16 Kundu GC, Mukherjee AB. Evidence that porcine pancreatic phospholipase A_2 via its high affinity receptor stimulates extracellular matrix invasion by normal and cancer cells. *J Biol Chem* 1997; 272: 2346–2353.
17 Pruzanski W, Vadas P, Browning J. Secretory non-pancreatic group II phospholipase A_2: role in physiologic and inflammatory processes. *J Lipid Mediat* 1993; 8: 161–167.
18 Qu XD, Lehrer RI. Secretory phospholipase A_2 is the principal bactericide for staphylococci and other gram-positive bacteria in human tears. *Infect Immunol* 1998; 66: 2791–2797.
19 Lambeau G, Lazdunski M. Receptors for a growing family of secreted phospholipases A_2. *Trends Pharmacol Sci* 1999; 20: 162–170.
20 Murakami M, Nakatani Y, Atsumi G, Inoue K, Kudo I. Regulatory functions of phospholipase A_2. *Crit Rev Immunol* 1997; 17: 225–283.
21 Fenard D, Lambeau G, Valentin E, Lefebvre J, Lazdunski M, Doglio A. Secreted phospholipases A_2, a new class of HIV inhibitors that block virus entry into host cells. *J Clin Invest* 1999; 104: 611–618.
22 Tischfield JA. A reassessment of the low molecular weight phospholipase A_2 gene family in mammals. *J Biol Chem* 1997; 272: 17247–17250.
23 Cupillard L, Koumanov K, Mattéi MG, Lazdunski M, Lambeau G. Cloning, chromosomal mapping, and expression of a novel human secretory phospholipase A_2. *J Biol Chem* 1997; 272: 15745–15752.
24 Valentin E, Ghomashchi F, Gelb MH, Lazdunski M, Lambeau G. On the diversity of secreted phospholipases A_2: cloning, tissue distribution, and functional expression of 2 novel mouse group II enzymes. *J Biol Chem* 1999; 274: 31195–31202.
25 Valentin E, Koduri RS, Scimeca J-C et al. Cloning and recombinant expression of a novel mouse-secreted phospholipase A_2. *J Biol Chem* 1999; 274: 19152–19160.
26 Suzuki N, Ishizaki J, Yokota Y et al. Structures, enzymatic properties, and expression of novel human and mouse secretory phospholipase A_2s. *J Biol Chem* 2000; 275: 5785–5793.
27 Ishizaki J, Suzuki N, Higashino K-C et al. Cloning and characterization of novel mouse and human secretory phospholipase A_2s. *J Biol Chem* 1999; 274: 24973–24979.
28 Valentin E, Lambeau G. Increasing molecular diversity of secreted phospholipases A_2 and their receptors and binding proteins. *Biochim Biophys Acta* 2000; 1488: 59–70.
29 Valentin E, Ghomashchi F, Gelb MH, Lazdunski M, Lambeau G. Novel human secreted phospholipase A_2 with homology to the group III bee venom enzyme. *J Biol Chem* 2000; 275: 7492–7496.
30 Gelb MH, Valentin E, Ghomashchi F, Lazdunski M, Lambeau G. Cloning and recombinant expression of a structurally

Cambridge Books Online © Cambridge University Press, 2009

novel human secreted phospholipase A_2. *J Biol Chem* 2000; 275: 39823–39829.
31 Leslie CC. Properties and regulation of cytosolic phospholipase A(2). *J Biol Chem* 1997; 272: 16709–16712.
32 Nalefski EA, Sultzman LA, Martin DM et al. Delineation of two functionally distinct domains of cytosolic phospholipase A_2, a regulatory Ca^{2+}-dependent lipid-binding domain and a Ca^{2+}-independent catalytic domain. *J Biol Chem* 1994; 269: 18239–18249.
33 Dessen A. Structure and mechanism of human cytosolic phospholipase A(2). *Biochim Biophys Acta* 2000; 1488: 139–148.
34 Gijon MA, Spencer DM, Kaiser AL, Leslie CC. Role of phosphorylation sites and the C2 domain in regulation of cytosolic phospholipase A_2. *J Cell Biol* 1999; 145: 1219–1232.
35 Hanel AM, Schuttel S, Gelb MH. Processive interfacial catalysis by mammalian 85-kilodalton phospholipase A_2 enzymes on product-containing vesicles: application to the determination of substrate preferences. *Biochemistry* 1993; 32: 5949–5958.
36 Glover S, de Carvalho M, Bauburt T et al. Translocation of the 85-kDa phospholipase A_2 from cytosol to the nuclear envelope in rat basophilic leukemia cells stimulated with calcium ionophore or IgE/antigen. *J Biol Chem* 1995; 270: 15359–15367.
37 Schievella AR, Regier MK, Smith WL, Lin LL. Calcium-mediated translocation of cytosolic phospholipase A_2 to the nuclear envelope and endoplasmic reticulum. *J Biol Chem* 1995; 270: 30749–30754.
38 Nakatani Y, Tanioka T, Sunaga S, Murakami M, Kudo I. Identification of a cellular protein that functionally interacts with the C2 domain of cytosolic phospholipase $A_2\alpha$. *J Biol Chem* 2000; 275: 1161–1168.
39 Uozumi N, Kume K, Nagase T et al. Role of cytosolic phospholipase A_2 in allergic response and parturition. *Nature* 1997; 390: 618–622.
40 Bonventre JV, Huang Z, Taheri MR et al. Reduced fertility and postischaemic brain injury in mice deficient in cytosolic phospholipase A_2. *Nature* 1997; 390: 622–625.
41 Sapirstein A, Bonventre JV. Specific physiological roles of cytosolic phospholipase A(2) as defined by gene knockouts. *Biochim Biophys Acta* 2000; 1488: 139–148.
42 Hirabayashi T, Shimizu T. Localization and regulation of cytosolic phospholipase A(2). *Biochim Biophys Acta* 2000; 1488: 124–138.
43 Pickard RT, Strifler BA, Kramer RM, Sharp JD. Molecular cloning of two new human paralogs of 85-kDa cytosolic phospholipase A_2. *J Biol Chem* 1999; 274: 8823–8831.
44 Song C, Chang XJ, Bean KM, Proia MS, Knopf JL, Kriz RW. Molecular characterization of cytosolic phospholipase A_2-beta. *J Biol Chem* 1999; 274: 17063–17067.
45 Balsinde J, Dennis EA. Function and inhibition of intracellular calcium-independent phospholipase A_2. *J Biol Chem* 1997; 272: 16069–72.
46 Ramanadham S, Hsu FF, Bohrer A, Ma Z, Turk J. Studies of the role of group VI phospholipase A_2 in fatty acid incorporation, phospholipid remodeling, lysophosphatidylcholine generation, and secretagogue-induced arachidonic acid release in pancreatic islets and insulinoma cells. *J Biol Chem* 1999; 274: 13915–13927.
47 Atsumi G, Murakami M, Kojima K, Hadano A, Takima M, Kudo I. Distinct roles of two intracellular phospholipase A_2s in fatty acid release in the cell death pathway. Proteolytic fragment of type IVA cytosolic phospholipase A_2alpha inhibits stimulus-induced arachidonate release, whereas that of type VI Ca^{2+}-independent phospholipase A_2 augments spontaneous fatty acid release. *J Biol Chem* 2000; 275: 18248–18258.
48 Larsson PK, Claesson HE, Kennedy BP. Multiple splice variants of the human calcium-independent phospholipase A_2 and their effect on enzyme activity. *J Biol Chem* 1998; 273: 207–214.
49 Mancuso DJ, Jenkins CM, Gross RW. The genomic organization, complete mRNA sequence, cloning, and expression of a novel human intracellular membrane-associated calcium-independent phospholipase A(2). *J Biol Chem* 2000; 275: 9937–9945.
50 Kramer RM, Hession C, Johansen B et al. Structure and properties of a human non-pancreatic phospholipase A_2. *J Biol Chem* 1989; 264: 5768–5775.
51 Horigome K, Hayakawa M, Inoue K. Purification and characterization of phospholipase A_2 released from rat platelets. *J Biochem* 1987; 101: 625–631.
52 Mizushima H, Kudo I, Horigome K et al. Purification of rabbit platelet secretory phospholipase A_2 and its characteristics. *J Biochem* 1989; 105: 520–525.
53 Aarsman AJ, Leunissen-Bijvelt J, Van den Koedijk CDMA, Neys FW, Verkleij AJ, Van den Bosch H. Phospholipase A_2 activity in platelets. *J Lipid Mediators* 1989; 1: 49–61.
54 Murakami M, Kudo I, Inoue K. In vivo release and clearance of rat platelet phospholipase A_2. *Biochim Biophys Acta* 1989; 1005: 270–276.
55 Sharp JD, White DL, Chiou XG et al. Molecular cloning and expression of human Ca^{2+}-sensitive cytosolic phospholipase A_2. *J Biol Chem* 1991; 266: 14850–14853.
56 Kim DK, Suh PG, Ryu SH. Purification and some properties of a phospholipase A_2 from bovine platelets. *Biochem Biophys Res Commun* 1991; 174: 189–196.
57 Kim DK, Kudo I, Inoue K. Purification and characterization of rabbit platelet cytosolic phospholipase A_2. *Biochim Biophys Acta* 1991; 1083: 80–88.
58 Street IP, Lin H-K, Laliberte F et al. Slow- and tight-binding inhibitors of the 85-kDa human phospholipase A_2. *Biochemistry* 1993; 32: 5935–5940.
59 Ghomashchi F, Loo R, Balsinde J et al. Trifluoromethyl ketones and methyl fluorophosphonates as inhibitors of group IV and VI phospholipases A_2: structure-function studies with vesicle, micelle, and membrane assays. *Biochim Biophys Acta* 1999; 1420: 45–56.
60 Ackermann EJ, Conde FK, Dennis EA. Inhibition of macrophage Ca(2+)-independent phospholipase A_2 by bromoenol

Cambridge Books Online © Cambridge University Press, 2009

lactone and trifluoromethyl ketones. *J Biol Chem* 1995; 270: 445–50.

61 Bartoli F, Lin H-K, Ghomashchi F, Gelb MH, Jain MK, Apitz-Castro R. Tight binding inhibitors of 85-kDa phospholipase A_2 but not 14-kDa phospholipase A_2 inhibit release of free arachidonate in thrombin-stimulated human platelets. *J Biol Chem* 1994; 269: 15625–15624.

62 Riendeau D, Guay J, Weech PK et al. Arachidonyl trifluoromethyl ketone, a potent inhibitor of 85-kDa phospholipase A_2, blocks production of arachidonate and 12-hydroxyeicosatetraenoic acid by calcium ionophore-challenged platelets. *J Biol Chem* 1994; 269: 15619–15624.

63 Kramer RM, Roberts EF, Manetta JV, Hyslop PA, Jakubowski JA. Thrombin-induced phosphorylation and activation of Ca^{2+}-sensitive cytosolic phospholipase A_2 in human platelets. *J Biol Chem* 1993; 268: 26796–26804.

64 Kramer RM, Roberts EF, Manetta JV, Sportsman JR, Jakubowski JA. Ca(2+)-sensitive cytosolic phospholipase A_2 ($cPLA_2$) in human platelets. *J Lipid Mediat* 1993; 6: 209–216.

65 Hefner Y, Börsch-Haubold AG, Wilde JI et al. Serine-727 phosphorylation and activation of cytosolic phospholipase A_2 by MNK1-related protein kinases. *J Biol Chem* 2000; 275: 37542–37551.

66 Nucciarelli F, Gresele P, Nardicchi V et al. Evidence that cytosolic phospholipase A_2 is down-regulated by protein kinase C in intact human platelets stimulated with fluoroaluminate. *FEBS Lett* 1999;450: 39–43.

67 Silk S, Clejan S, Witkom K. Evidence of GTP-binding protein regulation of phospholipase A_2 activity in isolated human platelet membranes. *J Biol Chem* 1989;264: 21466–21469.

68 Halenda SP, Banga HS, Zavoico GB, Lau LF, Feinstein MB. Synergistic release of arachidonic acid from platelets by activators of protein kinase C and Ca^{2+} ionophores. Evidence for the role of protein phosphorylation in the activation of phospholipase A_2 and independence from the Na+/H+ exchanger. *Biochemistry* 1989; 28: 7356–7363.

69 Akiba S, Sato T, Fujii T. Involvement of a guanine-nucleotide-binding protein-mediated mechanism in the enhancement of arachidonic acid liberation by phorbol 12-myristate 13-acetate and Ca^{2+} in saponin-permeabilized platelets. *Biophys Biochim Acta* 1990;1044: 291–296.

70 Pasquet JM, Quek L, Stevens C et al. Phosphatidylinositol 3,4,5-trisphosphate regulates Ca(2+) entry via Btk in platelets and megakaryocytes without increasing phospholipase C activity. *EMBO J* 2000; 19: 2793–2802.

71 Carey F, Menashi S, Crawford N. Location of cyclooxygenase and thromboxane synthetase in human platelet intracellular membranes. *Biochem J* 1982; 204: 847 –851.

72 van Willigne G, Gorter G, Akkerman J-WN. Thrombopoietin increases platelet sensitivity to a-thrombin via activation of the ERK2-$cPLA_2$ pathway. *Thromb Haemost* 2000; 83: 610–616.

73 Rodriguez-Linares B, Watson S. Thrombopoietin potentiates activation of human platelets in association with JAK2 and TYK2 phosphorylation. *Biochem J* 1996; 316: 93–98.

74 Lin LL, Wartmann M, Lin AY, Knopf JL, Seth A, Davis RJ. $cPLA_2$ is phosphorylated and activated by MAP kinase. *Cell* 1993; 72: 269–278.

75 de Carvalho MGS, McCormack AL, Olson E et al. Identification of phosphorylation sites of human 85-kDa cytosolic phospholipase A_2 expressed in insect cells and present in human monocytes. *J Biol Chem* 1996; 271: 6987–6997.

76 Borsch-Haubold AG, Bartoli F, Asselin J et al. Identification of the phosphorylation sites of cytosolic phospholipase A_2 in agonist-stimulated human platelets and HeLa cells. *J Biol Chem* 1998; 273: 4449–4458.

77 Flati V, Haque SJ, Williams BR. Interferon-alpha-induced phosphorylation and activation of cytosolic phospholipase A_2 is required for the formation of interferon-stimulated gene factor three. *EMBO J* 1996; 15: 1566–1571.

78 Borsch-Haubold AG, Kramer RM, Watson SP. Phosphorylation and activation of cytosolic phospholipase A_2 by 38-kDa mitogen-activated protein kinase in collagen-stimulated human platelets. *Eur J Biochem* 1997; 245: 751–9.

79 Borsch-Haubold AG, Kramer RM, Watson SP: Cytosolic phospholipase A_2 is phosphorylated in collagen-and thrombin-stimulated human platelets independent of protein kinase C and mitogen activated protein kinase. *J Biol Chem* 1995; 270: 25885–25892.

80 Borsch-Haubold AG, Kramer RM, Watson SP: Inhibition of mitogen-activated protein kinase does not impair primary activation of human platelets. *Biochem J* 1996; 318: 207–212.

81 Kramer RM, Roberts EF, Um SL, Borsch-Haubold AG, Watson SP, Fisher MJ, Jakubowski JA: p38 map kinase phosphorylates $cPLA_2$ in thrombin-stimulated platelets. *J Biol Chem* 1996; 271: 27723–27729.

82 Li Z, Jiang Y, Ulevitch RJ, Han J. The primary structure of p38γ: a new member of the p38 group of MAP kinases. *Biochem Biophys Res Commun* 1996; 228: 334–340.

83 Mertens S, Craxton M, Goedert M. SAP kinase-3, a new member of the family of mammalian stress-activated protein kinases. *FEBS Lett* 1996; 383: 273–276.

84 Cuenda A, Cohen P, Buee-Scherrer V, Goedert M. Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6): comparison of the specificities of SAPK3 and SAPK2 (RK/p38). *EMBO J* 1997; 16: 295–305.

85 Goedert M, Cuenda A, Craxton M, Jakes R, Cohen P. Activation of the novel stress-activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6); comparison of its substrate specificity with that of other SAP kinases. *EMBO J* 1997; 16: 3563–3571.

86 Cohen P, Goedert M. Engineering protein kinases with distinct nucleotide specificities and inhibitor sensitivities by mutation of a single amino acid. 1998; 5: R161–R164.

87 Buschbeck M, Ghomashchi F, Gelb MH, Watson SP, Borsch-Haubold AG. Stress stimuli increase calcium-induced arachidonic acid release through phosphorylation of cytosolic phospholipase A_2. *Biochem J* 1999; 344: 359–366.

Cambridge Books Online © Cambridge University Press, 2009

88 Fukunaga R, Hunter T. MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *EMBO J* 1997; 16: 1921–1933.

89 Waskiewicz AJ, Flynn A, Proud CG, Cooper JA. Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *EMBO J* 1997; 16: 1909–1920.

90 New L, Jiang Y, Zhao M et al. PRAK, a novel protein kinase regulated by the p38 MAP kinase. *EMBO J* 1998; 17: 3372–3384.

91 Deak M, Clifton AD, Lucocq JM, Alessi DR. Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. *EMBO J* 1998; 17: 4426–4441.

92 Ludwig S, Engel K, Hoffmeyer A et al. 3pK, a novel mitogen-activated protein (MAP) kinase-activated protein kinase, is targeted by three MAP kinase pathways. *Mol Cell Biol* 1998; 12: 6687–6697.

93 Rouse J, Cohen P, Trigon S et al. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell* 1994; 78: 1027–1037.

94 Waskiewicz AJ, Johnson JC, Penn B, Mahalingam M, Kimball SR, Cooper JA. Phosphorylation of the cap-binding protein eukaryotic translation initiation factor 4E by protein kinase Mnk1 in vivo. *Mol Cell Biol* 1999; 19: 1871–1880.

95 Currie S, Roberts EF, Spaethe SM, Roehm NW, Kramer RM. Phosphorylation and activation of Ca^{2+}-sensitive cytosolic phospholipase A_2 in MCII mast cells mediated by high-affinity Fc receptor for IgE. *Biochem J* 1994; 304: 923–928.

96 Hayakawa M, Kudo I, Tomita M, Inoue K. Purification and characterization of membrane-bound phospholipase A_2 from rat platelets. *J Biochem* 1988; 103: 263–266.

97 Mounier C, Faili A, Vargaftig BB, Bon C, Hatmi M. Secretory phospholipase A_2 is not required for arachidonic acid liberation during platelet activation. *Eur. J Biochem* 1993; 216: 169–175.

98 Kennedy BP, Payette P, Mudgett J et al. A natural disruption of the secretory group II phospholipase A_2 gene in inbred mouse strains. *J Biol Chem* 1995; 270: 22378–22385.

99 Asaoka Y, Yoshida K, Sasaki Y et al. Possible role of mammalian secretory group II phospholipase A_2 in T-lymphocyte activation: implication in propagation of inflammatory reaction. *Proc Natl Acad Sci USA* 1993; 90: 716–719.

100 Reddy ST, Herschman HR. Transcellular prostaglandin production following mast cell activation is mediated by proximal secretory phospholipase A_2 and distal prostaglandin synthase 1. *J Biol Chem* 1996; 271: 186–191.

101 Hara S, Kudo I, Inoue K. Augmentation of prostaglandin E_2 production by mammalian phospholipase A_2 added exogenously. *J Biochem* 1991; 110: 163–165.

102 Murakami M, Kudo I, Inoue K. Eicosanoid generation from antigen-primed mast cells by extracellular mammalian 14-kDa group II phospholipase A_2. *FEBS Lett* 1991; 294: 247–251.

103 Murakami M, Kudo I, Inoue K. Molecular nature of phospholipases A_2 involved in prostaglandin I2 synthesis in human umbilical vein endothelial cells. Possible participation of cytosolic and extracellular type II phospholipases A_2. *J Biol Chem* 1993; 268: 839–844.

104 Suga H, Murakami M, Kudo I, Inoue K. Exogenous type-II phospholipase A_2 stimulates prostaglandin synthesis in rat liver-derived BRL-3A cells in the presence of tumor necrosis factor α. *J Biochem* 1995; 118: 939–945.

105 Emadi S, Mirshahi M, Elalamy I, Nicolas C, Vargaftig BB, Hatmi M. Cellular source of human platelet secretory phospholipase A_2. *Br J Haematol* 1998; 100: 365–373.

106 Murakami M, Kambe T, Shimbara S, Yamamoto S, Kuwata H, Kudo I. Functional association of type IIA secretory phospholipase A_2 with the glycosyl phosphatidylinositol-anchored heparan sulfate proteoglycan in the cyclooxygenase-2-mediated delayed prostanoid biosynthetic pathway. *J Biol Chem* 1999; 274: 29927–29936.

107 Enomoto A, Murakami M, Kudo I. Internalization and degradation of type IIA phospholipase A(2) in mast cells. *Biochem Biophys Res Commun* 2000; 24: 667–672.

108 Fourcade O, Simon MF, Viode C et al. Secretory phospholipase A_2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells. *Cell* 1995; 80: 919–927.

109 Mounier C, Vargaftig BB, Franken PA, Verheij HM, Bon C, Touqui L. Platelet secretory phospholipase A_2 fails to induce rabbit platelet activation and to release arachidonic acid in contrast with venom phospholipases A_2. *Biochim Biophys Acta* 1994; 1214: 88–96.

110 Zachowski A. Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. *Biochem J* 1993; 294: 1–14.

111 Devaux PF. Static and dynamic lipid asymmetry in cell membranes. *Biochemistry* 1991; 30: 1163–1171.

112 Gelb MH, Cho W, Wilton DC. Interfacial binding of secreted phospholipases A_2: more than electrostatics and a major role for tryptophan. *Curr Opin Struct Biol* 1999; 9: 428–432.

113 Yokayama K, Kudo I, Inoue K. Phospholipid degradation in rat calcium ionophore-activated platelets is catalyzed mainly by two discrete secretory phospholipase *As. J Biochem* 1995; 117: 1280–1287.

114 Zwaal RFA, Comfurius P, Bevers EM. Platelet procoagulant activity and microvesicle formation – its putative role in hemostasis and thrombosis. *Biochim Biophys Acta* 1992; 1180: 1–8.

115 Ryningen A, Holmsem H. *Biochemistry* of platelet activation. In Rao GHR, ed: *Handbook of platelet physiology and pharmacology*. Kluwer academic publisher, 1999: 188–237.

116 Kroll MH, Schafer AIB. Biochemical mechanisms of platelet activation. *Blood* 1989; 74: 1181–1195.

117 Fujishima H, Sanchez-Mejia RO, Bingham CO et al. Cytosolic phospholipase A_2 is essential for both the immediate and the delayed phases of eicosanoid generation in mouse bone marrow-derived mast cells. *Proc Natl Acad Sci USA* 1999; 96: 4803–4807.

118 Kuwata H, Nakatani Y, Murakami M, Kudo I. Cytosolic

Cambridge Books Online © Cambridge University Press, 2009

phospholipase A_2 is required for cytosine-induced expression of type IIA secretory phospholipase A_2 that mediates optimal cyclooxygenase-2-dependent delayed prostaglandin E2 generation in rat 3Y1 fibroblasts. *J Biol Chem* 1998; 273: 1733–1740.

119 Andreani M, Olivier JL, Berenbaum F, Raymondjean M, Bereziat G. Transcriptional regulation of inflammatory secreted phospholipase A_2. *Biophys Biochem Acta* 2000; 1488: 149–158.

120 Laine VJO, Grass DS, Nevalainen TJ. Resistance of transgenic mice expressing human group II phospholipase A_2 to *Escherichia coli* infection. *Infect Immun* 2000; 68: 87–92.

121 Kaizer E. Phospholipase A_2: its usefulness in laboratory diagnostics. *Crit Rev Clin Lab Sci* 1999; 36: 65–163.

122 Pruzanski W, Scott K, Smith G, Rajkovid I, Stefanski E, Vadas P. Enzymatic activity and immunoreactivity of extracellular phospholipase A_2 in inflammatory synovial fluids. *Inflammation* 1992;16: 451–457.

123 Kortesuo PT, Nevalainen TJ. Phospholipase A_2 in human ascitic fluid. Purification, characterization and immunochemical detection. *Biochem J* 1991; 278: 263–267.

124 Cirino G, Cicala C, Sorrentino L, Browning JL. Human recombinant non pancreatic secreted platelet phospholipase A_2 has anticoagulant activity in vitro on human plasma. *Thromb Res* 1993; 70: 337–342.

125 Mounier CM, Hackeng TM, Schaeffer F, Faure G, Bon C, Griffin JH. Inhibition of prothrombinase by human secretory phospholipase A_2 involves binding to factor Xa. *J Biol Chem* 1998; 273: 23764–23772.

126 Inada M, Crowl RM, Bekkers A, Verheij H, Weiss J. Determinants of the inhibitory action of purified 14-kDa phospholipases A_2 on cell-free prothrombinase complex. *J Biol Chem* 1994; 269: 26338–26343.

127 Snitko Y, Koduri RS, Han SK et al. Mapping the Interfacial Binding Surface of Human Secretory Class IIa Phospholipase A_2. *Biochemistry* 1997; 36: 14325–14333.

128 Mounier CM, Luchetta P, Lecut C et al. Basic residues of human group IIA phospholipase A_2 are important for binding to factor Xa and prothrombinase inhibition. Comparison with other mammalian secreted phospholipases A_2. *Eur J Biochem* 2000; 267: 4960–4969.

129 Vadas P, Browning J, Edelson J, Pruzanski WJ. Extracellular phospholipase A_2 expression and inflammation: the relationship with associated disease states. *J Lipid Mediators* 1993; 8: 1–30.

130 Zwaal RFA, Schroit AJ. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood* 1997; 89: 1121–1132.

131 Atsumi G, Murakami M, Tajima M, Shimbara S, Hara N, Kudo I. The perturbed membrane of cells undergoing apoptosis is susceptible to type II secretory phospholipase A_2 to liberate arachidonic acid. *Biochim Biophys Acta* 1997; 1349: 43–54.

132 Nielson KH, Olsen CA, Allred DV, O'Neill KL, Burton GF, Bell JD. Susceptibility of S49 lymphoma cell membranes to hydrolysis by secretory phospholipase A_2 during early phase of apoptosis. *Biochim Biophys Acta* 2000; 1484: 163–174.

133 Hack CE, Wolbink GJ, Schalwijk C, Speijer H, Hermens WT, van den Bosch H. A role for secretory phospholipase A_2 and C-reactive protein in the removal of injured cells. *Immunol Today* 1997; 18: 111–115.

134 Phipps RP. Atherosclerosis: the emerging role of inflammation and the CD40-CD40 ligand system. *Proc Natl Acad Sci USA* 2000; 97: 6930–6932.

135 Maschberger P, Bauer M, Baumann-Siemon J et al. Mildly oxidized low density lipoprotein rapidly stimulates via activation of the lysophosphatidic acid receptor Src family and Syk tyrosine kinases and Ca^{2+} influx in human platelets. *J Biol Chem* 2000; 275: 19159–19166.

136 Hurt-Camejo E, Camejo G. Potential involvement of type II phospholipase A_2 in atherosclerosis. *Atherosclerosis* 1997; 132: 1–8.

137 Kovanen PT, Pentikainen MO. Secretory group II phospholipase A_2. A newly recognized acute-phase reactant with a role in atherogenesis. *Circ Res* 2000; 86: 610–612.

138 Schiering A, Menschikowski M, Mueller E, Jaross W. Analysis of secretory group II phospholipase A_2 expression in human aortic tissue in dependence on the degree of atherosclerosis. *Atherosclerosis* 1999; 144: 73–78.

139 Sartipy P, Camejo G, Svensson L, Hurt-Camejo E. Phospholipase A_2 modification of low density lipoproteins forms small high density particles with increased affinity for proteoglycans and glycosaminoglycans. *J Biol Chem* 1999; 274: 25913–25920.

140 Eckey R, Menschikowski M, Lattke P, Jaross W. Minimal oxidation and storage of low density lipoproteins result in an increased susceptibility to phospholipid hydrolysis by phospholipase A_2. *Atherosclerosis* 1997; 132: 165–176.

141 Sartipy P, Johansen B, Gåsvik K, Hurt-Camejo E. Molecular basis for the association of phospholipase A_2 Type IIa and decorin in human atherosclerotic lesions. *Circulation Res* 2000; 86: 707–714.

142 Ivandic B, Castellani LW, Wang XP et al. Role of group II secretory phospholipase A_2 in atherosclerosis. 1- Increased atherogenesis and altered lipoproteins in transgenic mice expressing group IIa phospholipase A_2. *Arterioscler Thromb Vasc Biol* 1999; 19: 1284–1290.

143 Caslake MJ, Packard CJ, Suckling KE, Holmes SD, Chamberlain P, Macphee CH. Lipoprotein-associated phospholipase A_2, platelet-activating factor acetylhydrolase: a potential new risk for coronary artery disease. *Atherosclerosis* 2000; 150: 413–419.

144 Foreman-Wykert AK, Weinrauch Y, Elsbach P, Weiss J. Cell-wall determinants of the bactericidal action of group IIA phospholipase A_2 against Gram-positive bacteria. *J Clin Invest* 1999; 103: 715–721.

145 Buckland AG, Wilton DC. The antibacterial properties of secreted phospholipases A_2. *Biophys Biochem Acta* 2000; 1488: 71–82.

Cambridge Books Online © Cambridge University Press, 2009

146 Weiss J, Inada M, Elsbach P, Crowl RM. Structural determinants of the action against *Escherichia coli* of a human inflammatory fluid phospholipase A_2 in concert with polymorphonuclear leukocytes. *J Biol Chem* 1994; 269: 26331–26337.
147 Laine VJO, Grass DS, Nevalainen TJ. Protection by group II phospholipase A_2 against *Staphylococcus aureus*. *J Immunol* 1999; 162: 7402–7408.
148 Harwig SS, Tan L, Qu XD, Cho Y, Eisenhauer PB, Lehrer RI. Bactericidal properties of murine intestinal phospholipase A_2. *J Clin Invest* 1995; 95: 603–610.

Cambridge Books Online © Cambridge University Press, 2009

16

Roles of phospholipase C and phospholipase D in receptor-mediated platelet activation

Mauro Torti[1] and Eduardo G. Lapetina[2]

[1]Department of Biochemistry, University of Pavia, Italy

[2]Cato Research Ltd., Westpark Corporate Center, Durham, North Carolina, USA

Introduction

For more than 30 years, the inositol phospholipids and the activities of various phospholipases have been intimately associated with fundamental aspects of the stimulation of platelets. Works of various scientific groups led to the initial discoveries in the 1970s and 1980s that related the metabolism of phospholipids to the early platelet physiological responses such as shape change, secretion, and aggregation. In many instances, these findings were used to develop signal transduction concepts in many other biological systems. The products resulting from activation of these phospholipases represent myriad second messengers with relevant biological roles. Phospholipases and their substrates and products are central to the action of many receptors, transducers, and protein kinases, as well as to the mobilization of ions. This chapter describes the roles of phospholipase C and phospholipase D in signal transduction, and it discusses how these activities interplay in a network of pathways leading to physiological responses.

Overview of phospholipase C

Phosphoinositide-specific phospholipase C (PLC) plays an essential role in receptor-mediated platelet activation. The mammalian PLCs represent a family of Ca^{2+}-dependent enzymes showing a substrate preference for phosphatidylinositol 4,5-bisphosphate (PIP_2) over phosphatidylinositol 4-phosphate (PIP) or phosphatidylinositol (PI)[1]. Hydrolysis of PIP_2 by PLC leads to the generation of two important intracellular messengers: inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 is a soluble molecule that promotes the rapid release of Ca^{2+} from internal stores. Because of PLC-mediated IP_3 formation, the cytosolic concentration of Ca^{2+} increases rapidly and transiently and causes the activation of Ca^{2+}-dependent downstream effectors such as protein kinases and proteases. For additional details on IP_3-mediated Ca^{2+} mobilization in platelets, refer to Chapter 17 in this book. DAG is a membrane-bound lipid second messenger that mediates the translocation and activation of the serine/threonine protein kinase C (PKC). The role of PKC in platelet activation by extracellular agonists is discussed in Chapter 18.

In addition to generating the intracellular second messengers IP_3 and DAG, PIP_2 hydrolysis by PLC may have other important consequences. PIP_2 also represents the preferred substrate for a different lipid-metabolizing enzyme, phosphatidylinositol 3-kinase (PI–3K). This kinase is activated in agonist-stimulated platelets and catalyses the phosphorylation of PIP_2 at the 3-position of the inositol ring to generate the second messenger, phosphatidylinositol 3,4,5-trisphosphate (PIP_3)[2]. Therefore, PIP_2 hydrolysis by PLC reduces the substrate availability for PI-3K and, thus may affect signal transduction pathways initiated by this kinase. Moreover, PIP_2 in the plasma membrane is known to interact with profilin and gelsolin, two proteins involved in the regulation of actin polymerization. Hydrolysis of PIP_2 in agonist-stimulated platelets, for instance, liberates profilin, which, in turn, may take away monomeric actin from the sequestering protein thymosin $\beta 4$ to facilitate its polymerization into F-actin filaments[3]. Finally, hydrolysis of PIP_2 by PLC stimulates phosphoinositide turnover. Soluble IP_3 is rapidly dephosphorylated to the inactive inositol 1,4-bisphosphate by an IP_3 5-phosphatase and is then progressively converted to free inositol

by the sequential action of 4- and 1-phosphatases and used for the resynthesis of membrane inositol phospholipids.

An alternative route for IP_3 metabolism involves the transient phosphorylation by a specific 3-kinase to generate inositol 1,3,4,5-tetrakisphosphate (IP_4) before degradation by the above-mentioned phosphatases. Although IP_4 does not induce Ca^{2+} mobilization from internal stores, evidence indicates it may have different signalling functions, such as regulation of Ca^{2+} influx[4]. Moreover, a protein that binds with high affinity to IP_4 has been cloned and identified as a GTPase activating factor for the small GTP-binding proteins ras and rap1[5]. Although ras is almost undetectable in platelets, rap1b is highly expressed in these cells[6]. Therefore, IP_4 may represent an intracellular messenger playing a role in signal transduction leading to platelet activation.

DAG, the other second messenger produced by the action of PLC, is rapidly inactivated by phosphorylation to phosphatidic acid (PA) through a DAG kinase[7]. PA is a metabolic precursor for phospholipid biosynthesis, but it may also be involved in signal transduction. Although early works reported the ability of exogenous PA to induce platelet aggregation[8], its role as intracellular messenger is quite controversial. Nevertheless, deacylation of PA by a phospholipase A_2 enzyme generates lysophosphatidic acid (LPA), which has been clearly recognized as an efficient platelet agonist[9]. Because of the multitude of signalling pathways activated or modulated by the action of PLC, it is clear this enzyme plays a central role in cell activation.

Agonist-induced phospholipase C activation in platelets

Because so many effects are elicited by the action of PLC, the measurements of a wide variety of intracellular parameters can be used to monitor PLC activation in response to extracellular platelet agonists, allowing for identification of the stimuli that affect this enzyme. These parameters include direct measurement of IP_3 formation, mobilization of Ca^{2+}, accumulation of PA, and phosphorylation of pleckstrin. Measurement of IP_3, however, provides the clearest evidence for PLC activation.

Traditionally, IP_3 accumulation is evaluated upon separation by ion-exchange chromatography of soluble inositol phosphates from platelets metabolically labelled with ^3H-inositol. The main disadvantages of this technique are the low incorporation of ^3H-inositol into membrane phosphoinositides and the rather complex and time-consuming analyses of the samples. More recently, radiobinding assays for IP_3 measurement have been developed, and they are commercially available. These are simple and specific assays, but rather expensive.

Measuring the release of Ca^{2+} from internal stores is an alternative to direct IP_3 measurement: Ca^{2+} mobilization can be detected by using fluorescent, membrane-permeable indicators such as FURA 2-AM or FLUO 3-AM. When platelet stimulation is performed in the absence of extracellular calcium, the cytosolic increase of this ion exclusively reflects the IP_3-mediated release from internal stores.

In addition, the production of DAG can be used to detect agonist-induced PLC activation. DAG is rapidly phosphorylated to PA; therefore, the lipids extracted from ^{32}P-labelled platelets can be separated by thin-layer chromatography and visualized by autoradiography to measure the accumulation of PA. Similarly, separation of the total platelet proteins by electrophoresis on acrylamide gels allows the identification and quantification of the phosphorylation of pleckstrin, the 47-kDa protein substrate for DAG-activated PKC.

All these experimental approaches have been widely used to identify the platelet agonists able to promote activation of PLC. It emerged that almost all the agents able to induce platelet activation by binding to a membrane receptor were also able to stimulate the action of PLC. Among these are soluble platelet agonists that bind to seven transmembrane-domain receptors coupled to heterotrimeric GTP-binding proteins such as thrombin, PAF, vasopressin, serotonin, ADP, lysophosphatidic acid, and thromboxane A_2, as well as its stable analogue U46619[10]. The only exception is epinephrine; this molecule binds to the α_{2A}-adrenergic receptor on the platelet membrane, and as yet seems to be the only platelet agonist not able to promote PLC activation directly[11,12]. The ability of ADP to stimulate PLC in platelets has been controversial for years. Early reports documented the inability of this agonist to stimulate PIP_2 breakdown[13,14], but more recent works measuring IP_3 formation and Ca^{2+} mobilization from internal stores definitively recognize the activation of PLC in ADP-stimulated platelets[15,16]. Because all the agonists cited above bind to membrane receptors coupled to G-proteins, a role for these signal transducers in PLC activation was hypothesized quite early.

Separately from the role of signal transducers, a role for GTP-binding proteins in PLC activation in human platelets also emerged from the observations made by several investigators during the 1980s. These scientists noted that GTP and its nonhydrolysable analogue, GTP-γ-S, were able to stimulate PLC activity in isolated platelet membranes and in permeabilized platelets[17–19]. For many years, the identity of the G-protein mediating PLC activation in platelets

Cambridge Books Online © Cambridge University Press, 2009

remained unknown, and this entity was generically called Gp. Since then, incredible progress has been made, and we now have a more complete picture of the G-proteins responsible for PLC stimulation in platelets. Activation of PLC in platelets, however, is not limited to agonists acting through G-protein-coupled receptors. Some adhesive proteins, such as collagen and von Willebrand factor (vWF), are potent platelet agonists. Although collagen receptors (GP VI) and vWF receptors (GP Ib–IX–V complex) are not associated with GTP binding proteins, collagen and vWF are able to promote PLC activation[20,21]. Moreover, some plant lectins such as wheat germ agglutinin (WGA) and concanavalin A (ConA) stimulate platelets in association with PLC activation[22,23]. Even cross-linking of the low-affinity receptor for the Fc fragment of immunoglobulin G (FcγRIIA) on the platelet membrane triggers PLC activation[24]. Finally, hydrolysis of PIP_2 and formation of PA have been reported to occur as a consequence of platelet adhesion to immobilized fibrinogen[25], and a role for the fibrinogen receptor integrin $\alpha_{IIb}\beta_3$ on PLC activation has been documented based on the measurement of intracellular calcium mobilization and IP_3 formation[26,27]. Thus, the mechanism for PLC activation in platelets clearly involves multiple signalling pathways.

Because multiple signalling pathways were involved, purification, cloning, and characterization of PLC were clearly mandatory to clarify the mechanisms of agonist-induced activation of this enzyme. A number of studies using chromatographic resolution, detected the isolation of multiple forms of PLC from both platelet membrane and cytosol[28–31]. These works clearly indicate that PLC is not a single enzyme, but is present in platelets as different isoforms. Moreover, because most platelet PLC activity was found in the cytosol, it was clear that the mechanism to activate these enzymes required the translocation to the membrane, where the physiological substrates are located.

By the end of the 1980s, an incredible number of different PLC isoforms had been identified both in platelets and in nucleated cells, and the nomenclature of PLC isozymes was clearly in disarray. In 1989, after a systematic immunological analysis of most of the reported PLC isoforms, Sue Goo Rhee proposed a unified nomenclature, based on Greek letters, which is still used today[32]. According to this nomenclature, all the known PLC isoforms fall into three major classes referred to as PLCβ, PLCγ, and PLCδ.

The phospholipase C isozymes

It is now well known that each of the three major classes of PLC contains multiple subtypes sharing a high degree of sequence homology. Four different PLCβ subtypes have been identified: PLCβ1, PLCβ2, PLCβ3, and PLCβ4. Their molecular masses range from 130 to 150 kDa, and different spliced variants have been described. The PLCγ family includes two members, PLCγ1 and PLCγ2, and each has a molecular mass of about 145 kDa. The PLCδ family contains the subtypes PLCδ1, PLCδ2, PLCδ3, and PLCδ4, which are smaller than other isozymes (85–88 kDa). Therefore, at least ten different isoforms of PLC, encoded by separated genes, exist in living cells. Not all of them are expressed in human platelets, but members of each of the three classes have been described in platelets. Using specific antibodies for the four members of the PLCβ family, Nozawa's team reported the presence of PLCβ2 and PLCβ3 in a whole-platelet lysate[31]. PLCβ1 was hardly detectable, indicating that it does not represent a major PLC isozyme in these cells, and PLCβ4 was not revealed at all[31]. Immunological studies found both members of the PLCγ family (PLCγ1 and PLCγ2) to be present in human platelets[33,34]. Evidence for the presence of PLCδ isoforms was also reported[31], and subsequently identified as PLCδ1 and PLCδ3[35,36]. A quantitative immunological study of PLC isozymes separated upon fractionation of platelet extracts by high-performance liquid chromatography revealed that seven of the ten known PLC isoforms are present in platelets[35]. This study failed to detect PLCδ3 in platelets, but this isozyme was subsequently reported to be present in platelets at a concentration at least three times higher than PLCδ1[36]. Altogether, these data indicate that PLCγ2 and PLCβ2 are the predominant PLC isoforms present in platelets.

The division of the PLC isoforms into three major classes was made based on structural homology, but was later discovered also to reflect different mechanisms of enzyme activation. It is now very well known that members of the PLCβ family are selectively activated by GTP-binding proteins. All the α-subunits of the members of the Gq family of heterotrimeric GTP-binding proteins (αq, α11, α14, α15, and α16) have been found to activate PLCβ isozymes, but not PLCγ or PLCδ isozymes[37,38]. The main α-subunits of the Gq family expressed in human platelets are αq and α16; other members were not found[39,40]. The different α-subunits of the Gq family display similar potency in activating PLCβ isozymes, and therefore, they are probably interchangeable. By contrast, the members of the PLCβ family differ in their sensitivities to stimulation by α-subunits. For instance, the αq and α16 subunits activate PLCβ isoforms in the order PLCβ1 = PLCβ3 $\gg$ PLCβ2[41,42]. In light of this, we may assume that in human platelets, PLCβ3, although less abundant than PLCβ2, is probably the main target for activation through αq or α16.

In addition to being regulated by the α-subunits of the

Cambridge Books Online © Cambridge University Press, 2009

Gq family of heterotrimeric G-proteins, PLCβ isozymes are stimulated by activated $\beta\gamma$ dimers[43,44]. The activation of PLCβ isoforms by $\beta\gamma$ subunits proceeds in the order PLCβ3 > PLCβ2 > PLCβ1[45–47]. Therefore, PLCβ2, which is the most abundant PLCβ isozyme in platelets, is probably activated preferentially through a $\beta\gamma$-dependent mechanism, while PLCβ3 can be efficiently activated by the αq and α16 subunits and the $\beta\gamma$ subunits.

The members of the PLCγ family are typically activated in response to peptide growth factors and cytokines acting through membrane receptors with intrinsic tyrosine kinase activity or associated with intracellular tyrosine kinases[48]. Indeed, activation of PLCγ enzymes involves phosphorylation on tyrosine residues. In platelets, the only receptor for growth factors recognized as yet is the PDGF receptor, whose activation, however, seems to lead to platelet inhibition rather than stimulation[49]. Despite this, PLCγ isozymes are highly expressed in platelets, and the amount of PLCγ2 is almost sixfold higher than that of PLCβ2[35]. It is now very well documented that PLCγ isozymes are activated in response to a variety of agonists whose receptors are not linked to G-proteins, such as collagen, von Willebrand factor, and immunocomplexes promoting cross-linking of FcγRIIA. The mechanisms for PLCγ2 activation in platelets are discussed later in this chapter.

PLCδ isoforms are not activated either by classical G-proteins or by a tyrosine-kinase-dependent mechanism, and the modality by which these enzymes are coupled to membrane receptors is still poorly understood. Because the activity of PLCδ isozymes is more sensitive to Ca^{2+} than that of other isoforms, this class may be regulated by the cytosolic concentration of this ion[50]. Alternatively, a recently described new class of GTP-binding protein, termed Gh has been reported to bind to and activate PLCδ1[51]. Moreover, activation of purified PLCδ1 is also promoted by RhoGAP, a protein that stimulates the GTPase activity of the small G-protein rhoA[52]. Interestingly both Gh and rhoA are expressed in human platelets[53,54], but their involvement in the activation of PLCδ1 in these cells remains unclear.

Structure of phospholipase C isozymes

Analysis of the structure of the three families of PLC isozymes reveals interesting details and suggests possible strategies underlying their activation. All PLC isoforms have a modular organization and are composed of several distinct domains. At the N-terminus, all the PLC isozymes contain a pleckstrin homology (PH) domain, which is followed by four consecutive EF-hand motifs. The catalytic domain is composed of two regions, designated X and Y. In PLCδ and PLCβ, these two regions are separated by a short sequence of 50–70 aminoacids, but in PLCγ this linker region is much longer (about 400 amino acids) and is composed of two SH2-domains followed by an SH3-domain, which are all included within a second split PH domain. In all the PLC isozymes, the catalytic domain is followed by a C2 domain. Members of PLCβ present at the C-terminus a peculiar extension of about 400 amino acids. This extension is not present in other PLC isoforms. A scheme of the structure of PLC isozymes is given in Fig. 16.1. Only the three-dimensional structure for PLCδ1 has been resolved[55], and the information obtained about this isozyme is useful in predicting the organization of other isoforms.

The catalytic domain

The X and Y regions forming the catalytic domain of all the PLC isoforms are the most conserved sequences among mammalian isozymes. Structural data from PLCδ1 reveal that these two regions are organized three-dimensionally to form two halves of a catalytic unit that accommodates the substrate in a solvent-accessible cleft near the active site[55]. Co-crystallization of PLCδ1 with the product of the reaction, IP_3, reveals that all the groups of the polar head of the phosphoinositide substrate interact with the active site[55,56]. No space has been found to accommodate a possible phosphate group bound to the 3-position of the inositol ring, providing structural explanation for the known inability of PLC to hydrolyse the lipid product of PI-3K, phosphatidylinositol 3,4,5-trisphosphate[57]. The 2-hydroxyl group of IP_3 is coordinated with a Ca^{2+} ion present at the bottom of the active site, and this may represent the structural basis for the absolute calcium requirement of all mammalian PLC. A number of residues involved in the catalytic reaction have been identified by both structural and mutational analysis[55,58]. Corresponding residues are highly conserved in all the PLC isoforms, and it was reported that mutation of some of them in PLCγ1 abrogates enzyme activity[59].

The PH domain

A PH domain is present at the N-terminus of all the known mammalian PLC. It consists of a module of about 120 residues initially described in pleckstrin, the main platelet substrate for PKC, and subsequently found in more than 100 different proteins[60]. Although different PH domains share a very low sequence of similarity, these domains fold into a highly conserved three-dimensional architecture, consisting of seven antiparallel β-strands organized in a

Cambridge Books Online © Cambridge University Press, 2009

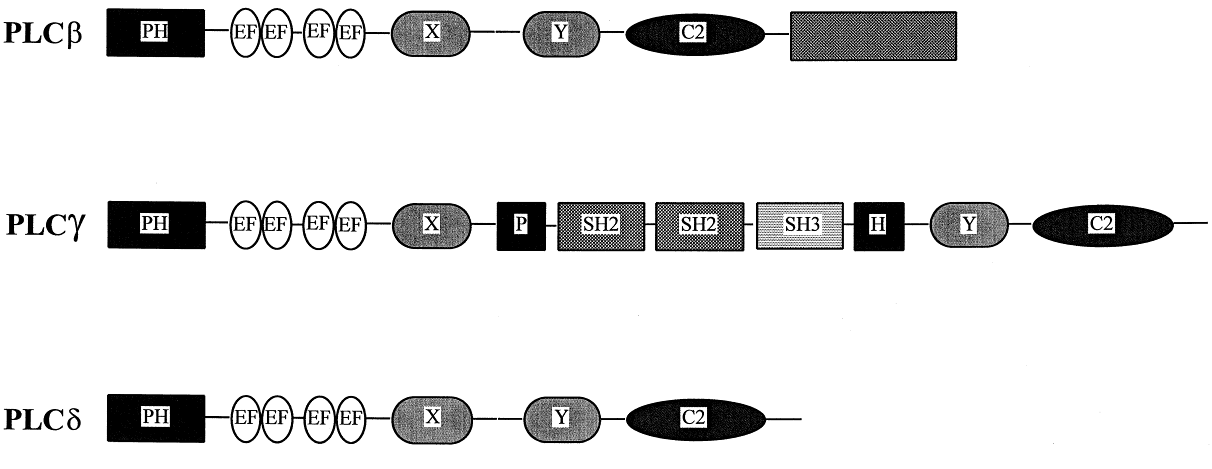


Fig.16.1. Multidomain structure of the three classes of PLC isozymes. All PLC isozymes contain an N-terminal PH domain that is involved, through different mechanisms, in membrane targeting. This domain is followed by four EF-hand motifs, which are Ca^{2+}-binding domains. The catalytic domains are named X and Y, and they are followed by a C2 domain, which is thought to act as a Ca^{2+}-dependent membrane lipid binding module. Unique to the PLCβ family of isozymes is a C-terminal tail that is involved in the binding of G-protein α-subunits. The PLCγ isozymes display an extended X/Y linker region that has essential regulatory functions and is composed of a second split PH domain, two SH2 domains, and one SH3 domain.

barrel-like structure. Because the entire PH domain is able to interact with membranes, this domain is thought to be involved in membrane targeting of proteins. As mentioned above, the evidence that most PLC activity in platelets is present in the cytosol and the finding that all PLC enzymes contain a PH domain suggest that this module may participate in the membrane translocation of PLC to allow access to phosphoinositide substrates. The crucial role of the PH domain in activation of PLC in platelets is supported by the evidence that, in these cells, pleckstrin, another PH-containing protein, can inhibit agonist-induced PIP_2 hydrolysis[61]. Although the role of PH domains in targeting proteins to the membrane is well documented, the mechanism by which this effect is achieved differs among the members of the PLC families of enzymes. In fact, the PH domains of PLCβ, PLCγ, and PLCδ display different binding specificities. The PH domain of PLCδ1, for instance, binds with high affinity to the PIP_2 of the membrane[62,63]. This interaction involves the polar head of the phospholipid, and, as expected, the PH domain of PLCδ1 can also bind to soluble IP_3. This suggests a possible mechanism for feedback regulation of PLCδ1 consisting of the displacement of the enzyme from the plasma membrane by the product of the reaction. The structural analysis of PLCδ1 PH domain allowed identification of the residues involved in PIP_2 binding and revealed that these residues are not conserved in the PH domains of members of the PLCβ and PLCγ families[64]. In fact, as expected, PH domains of these isozymes do not display affinity for PIP_2.

By contrast, the N-terminal PH domain of PLCγ has been found to bind to PIP_3, the lipid product of PI–3K[65]. The affinity of this interaction seems to be sufficient to support translocation of the protein to the membrane. Moreover, the SH2 domain of PLCγ also has been found to bind to PIP_3 with high affinity[66,67]. The level of 3-phosphorylated inositol lipids in resting cells is almost undetectable, but rapidly increases upon stimulation as a consequence of PI–3K activation. This evidence indicates that activation of PLCγ lies downstream of PI–3K activation. This is also supported by the finding that inhibitors of PI-3K, such as wortmannin, prevent activation of PLC in nucleated cells stimulated with growth factors[66,67] and in human platelets stimulated with collagen or by cross-linking of the FcγRIIA[68,69]. Moreover, in the latter case, wortmannin inhibition of PLC activation could be overcome by the addition of exogenous PIP_3[69]. The PH domain of PLCβ does not bind with suitable affinity to either PIP_2 or PIP_3[70]. In contrast, it has been shown that the PH domains of PLCβ1 and PLCβ2 interact strongly with the neutral lipid phosphatidylcholine[71]. Other PH domains, such as that of the β-adrenergic receptor kinase (βARK), bind G-protein $\beta\gamma$-subunits[72]. Such interactions could be relevant for those PLCβ isozymes that can be activated by $\beta\gamma$-dimers. Actually, the PH domain of PLCβ isozymes is able to promote the interaction of the proteins with $\beta\gamma$-dimers[73], and it is sufficient to stimulate enzyme activation[74]. Despite this, binding of $\beta\gamma$-subunits to PLCβ isozymes also involves sequences different from those found in the PH domain and located in the

Cambridge Books Online © Cambridge University Press, 2009

X/Y linker region[75,76]. Recent findings suggest that association of the PH domain of PLCβ isozymes with membrane phospholipids occurs independently of $\beta\gamma$-subunits, but facilitates lateral association of the enzyme with membrane-anchored $\beta\gamma$, leading to PLC activation[71].

The EF-hand motifs and the C2 domain

Four EF-hand motifs lying upstream of the catalytic region, and a C2 domain lying downstream, are common features of all PLC isozymes. Although their exact role in regulating PLC activity is not yet known, they appear to be involved in Ca^{2+} binding. Each of the EF-hand motifs consists of a helix–loop–helix motif similar to that found in calmodulin. Residues potentially able to interact with Ca^{2+} are present in these motifs, but direct evidence for binding of this ion has not been obtained[77]. Nevertheless, deletion of distinct portions of this region causes inactivation of both PLCδ1 and PLCγ1[78], which supports the hypothesis for an important role of the EF-hand motifs in regulating enzymatic activity.

The C2 domains are known to function as Ca^{2+}-dependent membrane lipid binding modules[79]. The C2 domain of PLCδ1 was actually found to be associated with three Ca^{2+} ions[77]. Although no binding studies have been reported as yet, it is likely that this domain is involved in membrane targeting of PLC isozymes.

The X/Y linker region

The region connecting the X and Y catalytic domains is essential for regulation of the activity of PLCγ isoforms. It contains two tyrosine residues that are phosphorylated concomitantly with enzyme activation (a third phosphorylated tyrosine is present in the C-terminal tail of PLCγ1, but is absent in PLCγ2). This region includes two SH2 and one SH3 domains, which are encompassed by a second split PH domain with unknown functions. The SH2 domains mediate protein-protein interaction by binding to tyrosine-phosphorylated proteins, and the SH3 domain specifically recognizes proline-rich sequences. It is very well known that the SH2 domains of PLCγ1 are responsible for the enzyme association with phosphorylated receptor for growth factors, and that this event represents the first step in efficient phosphorylation, membrane translocation, and activation of the enzyme[48]. In human platelets, the SH2 domains of PLCγ2 have been found to mediate the interaction with several signalling proteins[80]. In addition to tyrosine-phosphorylated proteins, the SH2 domains of PLCγ enzymes also specifically bind to PIP_3[66,67]. This interaction is thought to be essential for efficient translocation of the enzyme to the membrane. Because PLCγ isozymes are known to interact with PIP_3 also through the N-terminal PH domain, it appears clear that the lipid product of PI-3K plays a crucial role in regulating the activation of this family of enzymes[66,67]. The role of the SH3 domain of PLCγ is still poorly understood, partly because a physiological partner has not been identified. However, some evidence indicates that this domain may be involved in the translocation of PLCγ to the cytoskeleton[81].

Although very short in sequence, the X/Y linker region may also have regulatory functions in PLC isoforms different from those of PLCγ. For instance, it has been reported that proteolysis of PLCδ1 in the linker region results in the activation of the enzyme[82]. In members of the PLCβ family, the X/Y linker region may be involved in the binding of the regulatory $\beta\gamma$-subunits[76,83].

The C-terminal region

As mentioned earlier, members of the PLCβ family of enzymes are characterized by a C-terminal tail of about 400 amino acids that is absent in other PLC isoforms. Some studies indicate that this C-terminal tail is required for enzyme stimulation by Gα subunits[84,85]. Interestingly, deletion of the C-terminal tail of PLCβ2 results in an enzyme that is insensitive to stimulation by αq, but that can still be activated by $\beta\gamma$ dimers[86]. This means that the site of action of G-proteins α- and $\beta\gamma$-subunits is clearly distinct. Possible binding sites for $\beta\gamma$ on PLCβ have been localized in the X/Y linker region and in the PH domain[73–76], confirming the regulatory function of these sequences. The fact that α- and $\beta\gamma$-subunits of G-proteins activate PLCβ isozymes by binding to distinct regions may allow concomitant stimulation by both these signal transducers. Upon binding to the C-terminal tail of PLCβ, the intrinsic GTPase activity of αq increases by 100-fold. This indicates that the C-terminal tail of PLCβ acts as a GTPase activating protein (GAP) for αq[87].

Activation of phospholipase Cβ in platelets

To induce PLC activation, most of the soluble platelet agonists bind to seven transmembrane domains receptors coupled to heterotrimeric G-proteins. The only PLC isozymes regulated by G-proteins are the members of the PLCβ, and thus, these isoforms are candidates for activation by a large number of platelet agonists such as thrombin, TxA_2, and ADP. Although all four members of PLCβ family are present in platelets, the main isoforms are PLCβ2 and PLCβ3[35]. PLCβ1 is 40 times less abundant than

Cambridge Books Online © Cambridge University Press, 2009

PLCβ2, and PLCβ4 is 600 times less abundant than PLCβ2, and the amount of PLCβ2 is twice that of PLCβ3[35]. Recent evidence reports that PLCβ2 is present in platelets as two forms derived by alternative splicing, the difference being in 13 aminoacids in the C-terminal tail[88]. Platelets from a patient with a mild inherited bleeding disorder, showing reduced generation of IP_3, Ca^{2+} mobilization, and pleckstrin phosphorylation in response to different agonists, contained approximately one-third the amount of PLCβ2, suggesting a central role for this isozyme in platelet activation[35]. PLCβ isozymes are activated with different efficiency by the α-subunits of the Gq family of heterotrimeric G-proteins αq, α11, α14, α15, and α16[37,38]. Of these, only αq and α16 are expressed in human platelets[39,40]. Some studies have demonstrated that PLCβ2 is not significantly activated by αq or α16[41,42], therefore PLCβ3 is the only PLC isozyme that can be significantly activated by these α-subunits in platelets, and it also most likely represents the target of all the platelet agonists that signal through Gαq or Gα16.

The best-characterized example of the activation of PLCβ3 by these G-proteins is the agonist TxA_2 (Fig. 16.2(*a*)). Several studies based on copurification procedures, immunological evidence, and reconstitution experiments clearly demonstrated that the platelet TxA_2 receptor is coupled with αq to activate PLCβ3[39,89,90]. A possible association of the TxA_2 receptor with α16 has also been reported[91], and this may contribute to PLCβ3 activation in response to this agonist. The αq subunits are not substrates for pertussis toxin. Because PLC activation in response to TxA_2 is not affected by this toxin[92], the αq/α16-mediated activation of PLCβ3 probably accounts for the totality of PIP_2 hydrolysis promoted by TxA_2. This is consistent with the finding that inositol phosphates formation and calcium mobilization in response to TxA_2 are totally abolished in αq-deficient mice[93]. Moreover, these results implicitly question the physiological significance of a possible contribution of α16 in TxA_2-mediated PLC activation in platelets. Other G protein α-subunits such as α12 and α13 that were found to be associated with the TxA_2 receptor, do not seem to be involved in the signalling pathways leading to PLC activation[94].

Both members of the PLCβ family (PLCβ2 and PLCβ3) that are expressed in platelets at significant levels are efficiently activated by G-protein $\beta\gamma$-subunits[45–47]. In this regard, it is interesting to note that the efficiency of PLCβ3 stimulation by $\beta\gamma$ is almost twice that of PLCβ2[47]. This leads to two considerations. In platelets, PLCβ2 is probably activated mainly by $\beta\gamma$-subunits (because it is almost insensitive to α-subunits). Moreover, because the amount of PLCβ2 in platelets is almost twice that of PLCβ3, but the responsiveness of PLCβ3 to $\beta\gamma$-subunits is twice that of PLCβ2, agonists that liberate suitable $\beta\gamma$-dimers are most likely to lead to the activation of both PLCβ isozymes. Activation of PLCβ2 and PLCβ3 by $\beta\gamma$-dimers is thought to be the predominant mechanism for PLC activation in thrombin-stimulated platelets (Fig. 16.2(*b*)). It has been known for years that thrombin-induced hydrolysis of PIP_2 is inhibited by pertussis toxin[95]. The only G-protein α-subunit substrate for this toxin is αi, which is actually associated with the thrombin receptor but does not, as such, activate any PLC isozyme. However, thrombin-dependent activation of Gαi by GTP binding leads to the release of associated $\beta\gamma$-dimers that can be responsible for the pertussis toxin-sensitive activation of PLCβ2/PLCβ3. In this context, is has to be remembered that activation of PLCβ isozymes by $\beta\gamma$-dimers may not be limited to thrombin-stimulated platelets, but may potentially represent a general feature of all platelet agonists. In fact, in the absence of an exact map of association between specific α-subunits and $\beta\gamma$-dimers, we cannot exclude that PLC-activating $\beta\gamma$-dimers also may be associated with pertussis toxin-insensitive G-protein α-subunits, such as αq. In this regard, it is noteworthy that, as stated before, α-subunits of the Gq family and $\beta\gamma$-dimers activate PLCβ3 (the only isozyme sensitive to both stimulators in platelets) by binding to distinct domains, and therefore may synergistically contribute to the stimulation of this isozyme. Pretreatment of platelets with pertussis toxin inhibits, but does not totally abolish, PLC activation by thrombin, which, to a lesser extent, is still able to stimulate PIP_2 hydrolysis. This suggests the existence of a second pertussis toxin-insensitive pathway for PLC activation induced by this agonist. In addition to αi, also αq, α12, and α13 have been found to associate with the thrombin receptor in platelets[94–97]. Of these proteins, αq could mediate a pertussis toxin-insensitive activation of PLCβ3. Significantly, IP_3 formation and Ca^{2+} mobilization in response to thrombin are totally abolished in αq-deficient mice[93]. This observation suggests an essential role for αq in PLC activation by thrombin and reduces the physiological significance of the contribution of $\beta\gamma$-subunits to thrombin-dependent activation of PLCβ isozyme. However, these results clearly conflict with the concept that IP_3 formation by this agonist is produced, at least partially, by a mechanism sensitive to pertussis toxin that cannot be mediated by αq.

PLC activation in response to ADP is mediated through the binding of the agonist to P2Y1, one of the three receptors present on the platelet surface[15,16]. Despite the failure of early studies using a photoactivable GTP analogue to detect G αq among the G-proteins associated with the ADP receptors[98], it is now generally accepted that PLC activa-

Cambridge Books Online © Cambridge University Press, 2009

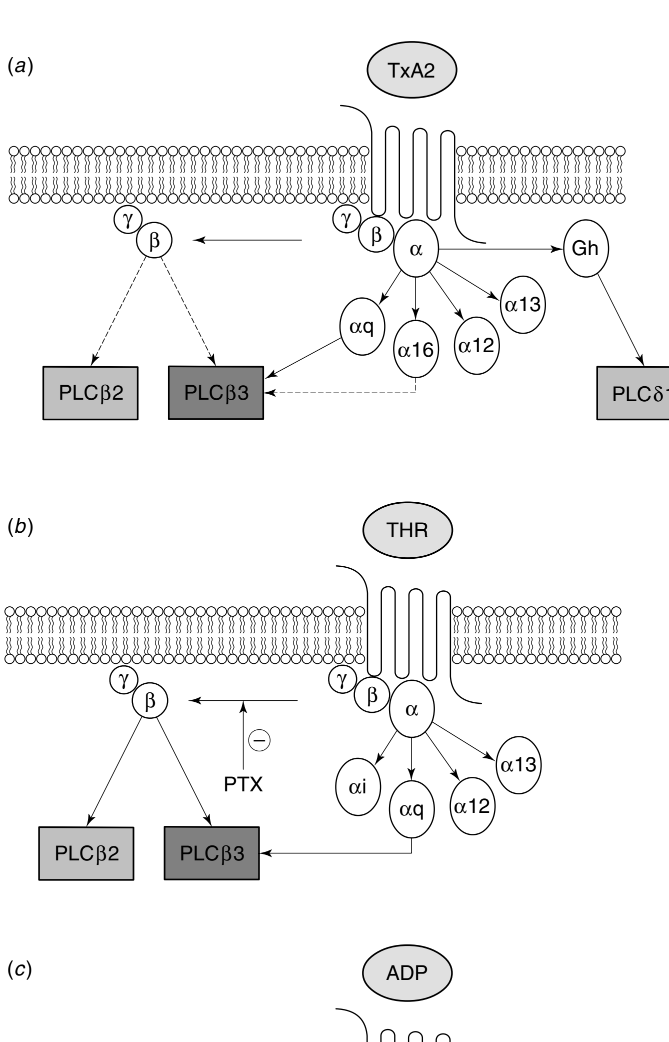


Fig. 16.2. G-protein-mediated activation of PLCβ isozymes in platelets. Although all four PLCβ isozymes are present in platelets, only PLCβ2 and PLCβ3 are expressed at significant levels[35]. PLCβ3 is efficiently activated by G-protein α-subunits, that, by contrast, are not efficient in activating PLCβ2[41,42]. G-protein $\beta\gamma$ dimers can activate both PLCβ2 and PLCβ3 with similar efficiency[45–47]. Solid lines indicate documented activating pathways, and dotted lines represent possible, although not yet confirmed mechanisms. (*a*) The platelet TxA_2 receptor is coupled to αq, α16, α12, and α13. Among these α-subunits, αq and α16 can activate PLCβ3[41,42]. Although the role of αq in PLC activation of TxA_2-stimulated platelets has been clearly documented[39,89,90,93], a direct involvement of α16 remains hypothetical. $\beta\gamma$ subunits released from activated α-subunits upon stimulation of the TxA_2 receptor can contribute to PIP_2 hydrolysis through both PLCβ2 and PLCβ3. The TxA_2 receptor is also coupled to the high molecular weight G-protein Gh[54], which has been reported to mediate activation of PLCδ1[51,54]. (*b*) The thrombin receptor is coupled to αi, αq, α12, and α13. αq has been demonstrated to be directly involved in thrombin-induced activation of PLC[93] by stimulating PLCβ3. However, PIP_2 hydrolysis induced by thrombin is inhibited by pertussis toxin[95], which catalyses the ADP-ribosylation of αi. Because αi does not activate any PLC isozyme, the pertussis toxin-sensitive activation of PLC is thought to be mediated by the action of $\beta\gamma$ dimers released from activated αi on PLCβ2 and PLCβ3. (*c*) Although many G-protein α-subunits have been found to associate with the seven transmembrane-domain ADP receptors, the only documented pathway for PLC activation involves the stimulation of PLCβ3 by αq[93]. However, an additional role for $\beta\gamma$ dimers, although not confirmed, cannot be ruled out.

tion by ADP is mediated by αq. This is based on the evidence that PLC activation by this agonist is totally abolished in αq-deficient platelets[93]. In this context, we may assume that PLCβ3 is the main PLC isozyme activated by ADP (Fig. 16.2(*c*)).

The activation of PLCβ3 may also be regulated by proteolytic cleavage of the enzyme. Stimulation of platelets with thrombin or collagen causes the degradation of PLCβ3 into a proteolytic fragment of about 100 kDa[99]. This proteolysis is also promoted by the Ca^{2+} ionophore A23187, and is prevented by calpeptin, suggesting that it is mediated by the Ca^{2+}- dependent protease, calpain. Based on immunological reactivity of the 100 kDa fragment, the site of cleavage on PLCβ3 has been tentatively localized between the Y region and the C-terminal tail. The 100-kDa proteolytic fragment of PLCβ3 is activated largely by $\beta\gamma$ subunits, unlike the intact protein[100]. Because calpain is activated by an aggregation-dependent mechanism, these observations

Cambridge Books Online © Cambridge University Press, 2009

suggest a novel strategy to amplify PLCβ3 activation, and this event may be related to irreversible aggregation.

Activation of phospholipase Cγ in platelets

Platelets express both members of the PLCγ family: PLCγ1 and PLCγ2. PLCγ2 is the most abundant protein of all the PLC isozymes in platelets, and its expression is tenfold higher than that of PLCβ2[35]. It is well accepted that PLCγ isozymes are activated by a mechanism involving the phosphorylation on PLCγ-tyrosine residues[48]. Therefore, initial studies undertaken to identify platelet agonists able to activate PLCγ focused on investigating the tyrosine phosphorylation of the two isozymes. Interestingly, tyrosine phosphorylation of PLCγ1 has never been detected in agonist-stimulated platelets, although this isozyme has been found to associate with tyrosine phosphorylated proteins[33,101–103]. One of these proteins has been identified as the GTPase-activation protein for p21ras or p120rasGAP, suggesting a link between PLCγ1 and monomeric GTP-binding proteins[33]. Although the lack of tyrosine phosphorylation of PLCγ1 raised concerns about its possible mechanism of activation in platelets, recent evidence indicates that it can be activated by some intracellular molecules, even in the absence of tyrosine phosphorylation[104]. For instance, PLCγ1 can be stimulated by arachidonic acid, which is also generated in platelets by the action of PLA_2 and by phosphatidic acid, produced by phospholipase D and by phosphorylation of PLC-released DAG. Although the exact mechanism and physiological relevance of PLCγ1 activation by these messengers in the absence of tyrosine phosphorylation remains unclear, these observations may indicate that PLCγ1 can be activated in platelets in response to different extracellular agonists.

More successful has been the investigation of tyrosine phosphorylation of PLCγ2 in stimulated platelets. This isozyme has been found to be phosphorylated in intact platelets stimulated with thrombin, collagen, von Willebrand factor, WGA, and concanavalin A, and by cross-linking of FcγRIIA[102,105–108]. The tyrosine phosphorylation of PLCγ2 in response to thrombin is very low compared with that observed in collagen- or von Willebrand factor-stimulated platelets, and the strongest phosphorylation of this isozyme is observed in concanavalin A-stimulated platelets. This evidence supports the idea that PLCγ2 may mediate PIP_2 hydrolysis in response to those agonists whose receptors are not coupled with GTP-binding proteins. Among these agonists, the most studied and best characterized example is collagen.

Collagen-induced tyrosine phosphorylation of PLCγ2 is mediated by binding to GPVI. This receptor has been recently cloned and belongs to the immunoglobulin superfamily[109,110]. It is a single transmembrane-domain glycoprotein expressed on the platelet surface in association with the Fc receptor γ-chain (FcR-γ chain), a signal-transducing unit containing an immunoreceptor tyrosine-based activation motif (ITAM) previously known as a component of several receptors for immune complexes[109–112]. The essential role of GPVI in collagen-induced PLCγ2 tyrosine phosphorylation is demonstrated by the fact that in GPVI-deficient platelets, phosphorylation of PLCγ2 by collagen is abolished[112]. Moreover, a cross-linked collagen-related triple-helical peptide (CRP) based on the repeated sequence Gly–Pro–Hyp, the snake venom protein convulxin and some monoclonal antibodies induce tyrosine phosphorylation of PLCγ2 by binding specifically to GPVI[113–115]. Tyrosine phosphorylation of PLCγ2 in response to these agonists is mediated by the tyrosine kinase Syk, as demonstrated both by studies with human platelets treated with a specific inhibitor of Syk, piceatannol, and by studies performed with platelets from Syk-deficient mice[116,117]. The signalling pathway leading to PLCγ2 tyrosine phosphorylation in collagen-stimulated platelets has been elucidated (Fig. 16.3). Collagen binding to the extracellular domain of GPVI results in the tyrosine phosphorylation of the associated FcR-γ chain, presumably within the ITAM[118]. Members of the Src family of tyrosine kinases Lyn and Fyn are probably involved in this initial step[119]. Tyrosine phosphorylated FcR-γ chain binds to the SH2 domain of the tyrosine kinase Syk leading to its activation and tyrosine phosphorylation[118]. Syk, in turn, mediates tyrosine phosphorylation of PLCγ2[116,117]. This mechanism seems to represent a general strategy for PLCγ2 phosphorylation in platelets. An ITAM is also present in the cytosolic domain of the FcγRIIA, and upon cross-linking of the receptor, is able to mediate the recruitment of Syk leading to tyrosine phosphorylation of PLCγ2[120]. Moreover, a role for FcγRIIA and the FcR-γ chain has also been proposed to be essential for PLCγ2 phosphorylation in von Willebrand factor-stimulated platelets[107,121].

The mechanism for tyrosine phosphorylation of PLCγ2, however, seems to be more complex than that depicted by the scheme illustrated above. For instance, coimmunoprecipitation experiments failed to detect a direct association between PLCγ2 and Syk, although a fusion protein containing the SH2 domains of PLCγ2 was found to precipitate Syk from a lysate of collagen-stimulated platelets[80]. This suggests that the interaction between Syk and PLCγ2 may be indirect, and that other factors may be required. In this context, a role for Bruton's tyrosine kinase (Btk) has been reported[122]. This kinase belongs to the Tec family of kinases

Cambridge Books Online © Cambridge University Press, 2009

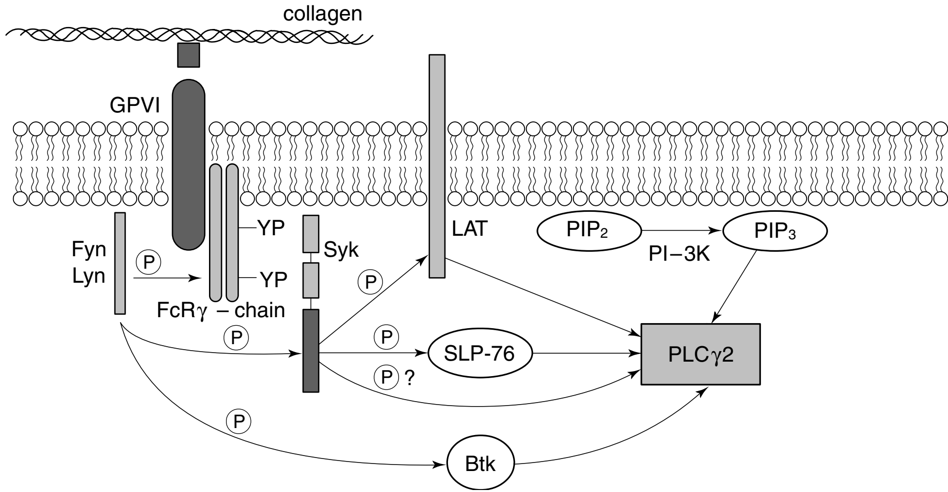


Fig. 16.3. PLCγ2 activation in collagen-stimulated platelets. Several studies performed with specific inhibitors or with selective knock-out mice have revealed that activation of PLCγ2, upon binding of collagen to the GPVI/Fc receptor γ-chain receptor on the platelet surface, requires the contribution of several signalling molecules, including the tyrosine kinases Fyn, Lyn, Syk, and Btk, the adaptor proteins LAT and SLP-76, and the lipid product of PI-3K, PIP_3. Syk and Btk may be involved in the tyrosine phosphorylation of PLCγ2, while LAT, SLP-76, and PIP_3 may participate in the translocation of PLCγ2 to the membrane. For details, see the text.

and is tyrosine phosphorylated in platelets stimulated with collagen or CRP. Btk is not expressed in platelets from patients affected with the X-linked agammaglobulinemia (XLA). Collagen stimulation of Btk-deficient XLA platelets results in an impaired tyrosine phosphorylation of PLCγ2 and Ca^{2+} mobilization[122].

In addition, two adaptor proteins, SLP-76 and LAT (linker for activation of T cells), seem also to be essential for PLCγ2 phosphorylation and activation. SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa) is a 76 kDa cytosolic protein that plays an essential role in PLCγ1 activation in T-cells[48]. In human platelets, SLP-76 is tyrosine phosphorylated in response to CRP by a mechanism depending on Syk[123]. Mutant mice lacking SLP-76 show fetal hemorrhage and prenatal mortality[124]. In SLP-76-deficient platelets, collagen-induced PLCγ2 tyrosine phosphorylation is totally abolished, and Ca^{2+} mobilization is strongly attenuated. Moreover, in vitro binding studies using a GST-fusion construct containing the two SH2 domains of PLCγ2 have documented a direct interaction between PLCγ2 and SLP-76[80]. These pieces of research clearly demonstrate that SLP-76 is an important link between Syk activation and PLCγ2 regulation, although the exact role played by this adaptor protein is still unclear.

LAT is a 38 kDa transmembrane protein involved in PLCγ1 activation in T cells[48]. In human platelets, LAT is tyrosine phosphorylated in response to both CRP and thrombin, by a mechanism depending on Syk but independent from Btk or SLP-76[125]. In platelets from mice lacking LAT, tyrosine phosphorylation of PLCγ2 and accumulation of phosphatidic acid were strongly reduced[125].

Altogether, these results indicate that LAT and SLP-76 are both regulated by Syk and are required for PLCγ2 tyrosine phosphorylation and activation induced by collagen. The fact that LAT is a membrane protein may suggest that it could also participate in the mechanism for PLCγ2 translocation to the membrane, which is essential for its activation. It is known that tyrosine phosphorylation of PLCγ isozymes is required but not sufficient for activation of the enzyme in the absence of an efficient translocation to the membrane. In this context, it is very well known that the lipid products of PI-3K play a crucial role in PLCγ2 translocation and activation. Studies from nucleated cells revealed that both the PH and the SH2 domains of PLCγ bind to PIP_3 to promote membrane translocation of the enzyme[65–67]. A similar role for the lipid products of PI-3K has also been proposed in human platelets[68,69]. PI-3K inhibitors like wortmannin or Ly294002 strongly reduced PLC activation in response to CRP or upon cross-linking of the FcγRIIA. These inhibitors did not prevent tyrosine phosphorylation of PLCγ2, but abolished its translocation to the particulate fraction. This effect was overcome by the addition of exogenous PIP_3 to permeabilized platelets[69]. Although platelet PLCγ2 was found to bind directly to PIP_3, the exact role of this interaction in mediating enzyme translocation to the platelet membrane is not totally clear.

Cambridge Books Online © Cambridge University Press, 2009

Recent evidence demonstrates that, when transfected into PC12 cells, the isolated PH or SH2 domains of PLCγ2 do not undergo membrane translocation upon elevation of PIP_3[126]. On the basis of this evidence, it has been proposed that interaction mediated by these domains may not be strong enough to ensure efficient translocation, but may help hold PLCγ2 at the membrane in association with other supporting interactions. In this regard, it is interesting to note that interaction of the SH2 domains of PLCγ2 with the membrane-localized adaptor protein LAT has been reported[80]. This supports the idea that the role of LAT in regulating PLCγ2 activation is related to membrane translocation.

In conclusion, activation of PLCγ2 in collagen-stimulated platelets clearly involves multiple regulators. It is possible that the basic scheme, which strongly resembles that described in B and T cells, represents a general strategy that might also operate in platelets stimulated with von Willebrand factor or by cross-linking of FcγRIIA, where the involvement of PLCγ2 has been reported. However, although incredible progress has been made during the last 3 years in understanding how collagen activates PLC, the exact mechanism of this process is yet to be fully clarified, and some discrepancies remain to be explained. For instance, platelet activation by collagen is totally inhibited in αq-deficient mice[93], and this convincing evidence hardly fits with the model proposed above. This evidence would be consistent with the activation of PLC in collagen-stimulated platelets being totally dependent on TxA_2 production. Early works actually reported that inositol phosphate accumulation induced by collagen is strongly inhibited by aspirin[127,128]. Moreover, PLC activation by collagen is also totally inhibited by cytochalasin D, highlighting a role for the intracellular cytoskeleton[128]. However, the steps that lead GPVI-FcR-γ to activate PLCγ2 do not include any role for the endogenous TxA_2 or cytoskeleton. Moreover, PLC activation by collagen has also been found to require the production of H_2O_2[129]. Alternatively, collagen also binds to integrin $\alpha_2\beta_1$ on the platelet surface, and the contribution of this receptor in cell activation is controversial[116,130,131]. Recently, it has been shown that tyrosine phosphorylation of PLCγ2 can also be promoted upon recruitment of integrin $\alpha_2\beta_1$[116,132]. The effects elicited through this collagen receptor, but not those mediated by GPVI, are markedly suppressed by aspirin or cytochalasin D[132]. These apparent discrepancies may be related to the doses of collagen used to stimulate platelets. The GPVI-dependent pathway for PLCγ2 activation has been elucidated in studies using high doses of collagen or CRP, and other findings come from studies in which much lower doses of agonist were used. It is therefore possible that the mechanism of PLC activation by collagen may depend upon the concentration of the agonist.

Activation of phospholipase Cδ in platelets

Although PLCδ1 and PLCδ3 are present in human platelets, their concentrations are much lower than other PLC isozymes[35,36]. PLCδ1 is as yet the only PLC isozyme whose structure has been defined; however, very little is known about the mechanism of activation of this class of enzymes. Although PLCδ1 is present mainly in the cytosol of platelets, PLCδ3 is predominantly located in the membrane fraction[36], suggesting the two isozymes may be regulated differently. The possibility that PLCδ isozymes are regulated by Ca^{2+} has been proposed[50], and in this context, it is possible that PLCδ1 and PLCδ3 are also stimulated in platelets in response to those agonists able to increase the intracellular concentration of Ca^{2+}. That is, all the platelet agonists that activate other PLC isozymes and stimulate IP_3-dependent Ca^{2+} release from internal stores could be responsible for a temporary delayed activation of PLCδ members. In addition, evidence indicates that PLCδ1 in platelets can be selectively activated in response to TxA_2. This is based on the observation that PLCδ1 is stimulated by Gh, a recently identified high molecular weight (70 kDa) G-protein that also displays transglutaminase activity[51] and that is expressed in human platelets[54]. Gh has been found to associate with both isoforms of the TxA_2 receptor (TPα and TPβ)[54]. In cells cotransfected with TPα, the main TxA_2 receptor expressed on the platelet surface, and Gh, the TxA_2 analogue U46619 was able to stimulate inositol phosphate accumulation in a manner similar to that measured in cells cotransfected with TPα and αq[54]. These results indicate that Gh could efficiently link the TxA_2 receptor to PLCδ1. Whether this mechanism is physiological relevant in human platelets remains to be clarified.

Phospholipase C and the platelet cytoskeleton

One of the most important events in platelet activation by extracellular agonists is the stimulation of actin polymerization and the reorganization of the intracellular cytoskeleton. It is now very well recognized that the platelet cytoskeleton does not have an exclusively contractile function, but also represents a network connecting and regulating several signalling molecules, including PLC. For instance, it has been shown that treatment of intact platelets with cytochalasin D, which prevents actin polymerization, blocks collagen-induced PLC activation[128]. Several

Cambridge Books Online © Cambridge University Press, 2009

actin-binding proteins may be involved in the regulation of PLC, including profilin and gelsolin. Profilin isolated from platelets has been found to bind with high affinity to PIP_2 and to compete efficiently with PLC for interaction with the substrate[133]. Moreover, profilin is thought to facilitate actin polymerization by subtracting actin monomers from the sequestering protein thymosin β4[3]. This process is activated when free profilin is released upon PIP_2 hydrolysis by PLC. Therefore, PLC and profilin may influence each other reciprocally.

Gelsolin is an F-actin severing protein. In platelets, a complex composed by gelsolin and actin in a 1: 1 ratio has been found to copurify and coimmunoprecipitate with PLCγ1[134]. Upon stimulation with thrombin, the actin–gelsolin complex dissociates from PLCγ1[135]. This event occurs concomitantly with platelet aggregation and is paralleled by a threefold to fivefold increase in PLCγ1 activity[135]. Therefore, it may represent a mechanism for a delayed, aggregation-dependent activation of this enzyme.

Upon platelet activation, several lipid-metabolizing enzymes have been found to associate physically with the actin-based cytoskeleton. These include PI-3K, DAG kinase, and PLC[136]. Among the PLC isozymes, PLCβ2, PLCβ3, PLCγ1, and PLCγ2 relocalize to the cytoskeleton in thrombin-stimulated platelets. Translocation of PLCγ1 is very rapid and transient and can be prevented by inhibitors of tyrosine kinases[137]. Translocation of PLCβ2, PLCβ3, and PLCγ2 to the cytoskeleton is blocked by inhibitors of fibrinogen binding and requires platelet aggregation[138]. Although PLCβ2 and PLCβ2 progressively accumulate in the cytoskeletal fraction during platelet aggregation, the interaction of PLCβ3 with the actin filaments is quicker but more transient. Because PLCβ3 is a substrate for calpain, which is activated during platelet aggregation[99], it is conceivable that the decreased cytoskeletal levels of this protein in the late phases of platelet activation reflect its hydrolysis. Although the exact consequence of PLC isozyme's interaction with the cytoskeleton is not known, it is possible that it represents a strategy to coordinate and facilitate activation of different isozymes in different phases of platelet activation. The investigation of the mechanisms regulating different PLC isozymes, and the clarification of the cellular events promoted by the activation of selective isoforms await the availability of specific inhibitors. The compound U73122 has been widely used to block the activity of both PLCβ and PLCγ isoforms[139,140]. However, recent studies raised questions about the specificity of this compound, which was shown to also affect Ca^{2+} influx and to prevent platelet aggregation by a mechanism independent of PLC inhibition[141,142].

Overview of phospholipase D

The role of the stimulation of phospholipase D (PLD) in receptor-mediated platelet activation is not as clearly defined as that of PLC. PLD hydrolyses the phosphate diester bond of membrane glycerophospholipids to produce PA and the free alcohol corresponding to the polar group[143]. Although PLD can act on phosphatidylethanolamine and phosphatidylinositol, the classical substrate for this enzyme is phosphatidylcholine (PC), which is hydrolysed to PA and choline. No obvious role for choline as an intracellular messenger is known. This molecule is rapidly phosphorylated to phosphocholine to be reused for PC biosynthesis. In contrast, PA may act as a second messenger by itself and represent a precursor for the synthesis of the potent lipid messenger, lysophosphatidic acid. Moreover, PA produced by the action of PLD can be converted to DAG by a PA-phosphohydrolase and, therefore, can lead to activation of PKC. In this way, PLD may contribute to and extend the regulation of PKC activity in response to extracellular agonists. Several studies have shown that in agonist-stimulated cells, the accumulation of DAG is biphasic: a first, rapid and transient, production of this messenger is followed by a second, more delayed, but sustained phase. It has been proposed that the first wave of DAG production is caused by PLC-mediated hydrolysis of PIP_2, and the second phase derives from the action of PLD on PC[144,145]. The PA-phosphohydrolase and DAG kinase activities create a bidirectional connection between PA and DAG, establishing a strong link between PLD and PLC (Fig. 16.4). Moreover, the close relationship between the two classes of enzymes is strengthened by at least two additional findings. First, PIP_2, the substrate of PLC, is also an essential cofactor for PLD (see below); second, the expression of a putative PLC enzyme that specifically acts on PC rather than PIP_2 (PC-PLC) to produce DAG and phosphocholine has been described in many cells, including platelets[146,147]. Because of these interplays with PLC, measurements of the direct products of the reaction may not always represent a reliable method to evaluate PLD activation in the living cell. Production of ^{3}H-choline from ^{3}H-choline-labelled cells may also be caused by the combined action of the putative PC-PLC and of phosphocholine hydrolase. Similarly, ^{3}H-phosphocholine is not only a metabolite that accumulates upon the combined action of PLD and choline kinase, but it is also directly produced by PC-PLC. In addition, a decrease in membrane PC, which may appear indicative for PLD activation, may be caused by PC-PLC activity. Alternately, even the measurement of ^{32}P-PA production in ^{32}P-labelled cells is not indicative of PLD activation because the turnover of the primary phosphate group in membrane phospholipids is very low, and accumulation of

Cambridge Books Online © Cambridge University Press, 2009

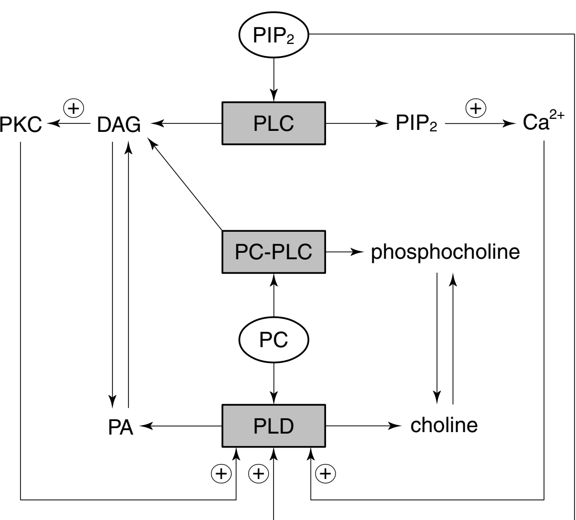


Fig. 16.4. Interplay between reactions catalysed by PLC and PLD. PC is hydrolysed by both PLD and PC-specific PLC. DAG and PA are interconverted by the action of PA-hydrolase and DAG kinase. Choline, produced by the action of PLD, is phosphorylated to phosphocholine, and phosphocholine generated by the PC-PLC is converted to choline. PIP_2 is an essential cofactor for PLD activation. DAG and IP_3, the products of PIP_2 hydrolysis by PLC, promote PKC activation and release of Ca^{2+} from intracellular stores. PKC and Ca^{2+} are known activators of PLD.

^{32}P-PA almost exclusively reveals the DAG kinase reaction on the product of PIP_2-specific PLC. The most direct and reliable approach to detect PLD activation exploits the unique ability of this enzyme to catalyze a transphosphatidylation reaction, in which the phosphatidyl group of PC is transferred not to a molecule of water, as in the normal hydrolysis reaction, but to a primary alcohol, such as ethanol or butanol. When the membrane phospholipids are labelled with ^{3}H-myristic acid or ^{3}H-palmitic acid, and cell stimulation is performed in the presence of ethanol or butanol, the accumulation of stable labelled phosphatidylethanol or phosphatidylbutanol represents unequivocal evidence for activation of PLD. The transphosphatidylation reaction, in addition to the measurement of accumulation of PA and choline, has been widely used to detect PLD activity in the cells and to measure PLD activity in vitro to clarify the mechanisms regulating this enzyme.

Regulation of phospholipase D

The PLD activity in intact cells can be regulated by a number of factors, including PIP_2, PKCα, and the low molecular weight GTP-binding proteins Arf, Rho, Rac, and Cdc42.

Regulation by PIP_2

In addition to being a substrate for PLC and PI-3K, PIP_2 is also involved in the regulation of PLD. This lipid cannot be considered a real activator of PLD, but rather represents an essential cofactor for optimal expression of the PLD enzymatic activity. Such a role for PIP_2 emerged from the casual finding that PLD activity measured in vitro using phospholipid vesicles containing labeled PC as a substrate was markedly increased by the inclusion of PIP_2[148]. Activation of PLD partially purified from brain membrane was increase tenfold by PIP_2, but not by other acid phospholipids, including PIP and PI. Studies with permeabilized cells confirm the physiological relevance of the regulatory role of PIP_2 on PLD activation in vivo[149]. In this experimental system, inhibition of PI 4-kinase reduced by 80% the amount of membrane PIP_2 and fully suppressed activation of PLD in response to GTPγS and PMA. Subsequent studies showed that, in addition to PIP_2, PIP_3, which is the lipid product of PI–3K, also can support stimulation of PLD[150]. The role of PIP_2 and PIP_3 as cofactors for PLD establishes a functional link among the lipid-metabolizing enzymes PLD, PLC, and PI-3K, providing the basis for a possible coordinated regulation of this signalling molecule.

Stimulation of phospholipase D by low molecular weight G-proteins

The involvement of G-proteins in regulating PLD was suggested based on the evidence that PLD was activated by GTP and by its non-hydrolysable analogue GTPγS[151,152]. Stimulation of PLD by GTP was found to be mediated by a cytosolic factor that, upon purification, was recognized as a member of the Arf family of proteins[148,153]. Arf proteins are low-molecular-weight GTPases initially identified as cofactors required for efficient ADP-ribosylation of the α-subunit of Gs by cholera toxin[154]. It has been subsequently demonstrated, and it is now very well accepted, that Arf proteins play an essential role in vesicular membrane trafficking in eukaryotic cells by promoting the formation of coated vesicles and facilitating the transport between the endoplasmic reticulum and the Golgi complex[155]. Interestingly, different studies have suggested a role for PLD in agonist-dependent cellular secretion, and abundant levels of Arf-sensitive PLD activity has been detected in the Golgi membranes[156,157]. Activation of PLD by Arf requires the N-terminal myristoylation of the small G-protein, a posttranslational modification leading to membrane anchorage[148], and is mediated by a domain located at the N-terminal region of the protein, distinct from the

Cambridge Books Online © Cambridge University Press, 2009

domain necessary for activation of cholera toxin[158]. Moreover, a direct interaction between Arf and PLD has been suggested[159].

In addition to Arf, other activators of PLD include members of the Rho family of small G-proteins (RhoA, RhoB, RhoC, Rac1, Rac2, and Cdc42). This was initially suggested by evidence that GTPγS activation of PLD could be potentiated by smgGDS, a protein that facilitates the GDP/GTP exchange (and thus the activation) of Rho, and could by inhibited by RhoGDI, which prevents activation of Rho[160,161]. Significantly, the inhibitory effect of rhoGDI on PLD activity could be overcome by addition of recombinant Rho proteins[161]. Moreover, stimulation of PLD activity was also inhibited by treatment of the cell with the botulinum toxin C3, which promotes ADP-ribosylation and inactivation of Rho[162]. Finally, partially purified PLD activity could be activated in vitro by recombinant Rho proteins[163]. Interestingly Rho proteins activate PLD in a PIP_2-dependent mechanism. The ability of Rho proteins to stimulate PLD requires the membrane localization of the Rho proteins, which, as in the case of Arf, is dependent upon the isoprenylation of the C-terminal region of the protein[163].

Activation of phospholipase D by PKC

In cell-free preparations, PLD activity can be stimulated in a dose-dependent manner by purified PKC[164]. The mechanism of PLD activation by PKC is not completely known. Although it is well accepted that the classical PKCs (α, β, and γ) but not the newer or the atypical isoforms, are effective stimulators of PLD in the presence of PMA or Ca^{2+}[165], the role of the catalytic function of these enzymes is controversial. In some experimental systems, ATP is required for PKC-dependent PLD activation, suggesting a mechanism involving protein phosphorylation[166]. However, in HL60 cells, stimulation of PLD by PKC requires ATP, but is not affected by inhibitors of the PKC activity[167]. Moreover, some studies have shown that PKCα with depleted kinase activity maintains the ability to stimulate PLD[168]. By limited proteolysis, the PLD-activating fragment of PKCα has been identified as the regulatory, but not the kinase domain. Although the exact mechanism for PLD activation by PKC is not clear, this regulatory mechanism establishes an additional link between PLD and PLC.

Structure of phospholipase D isozymes

Although much less is known about the identity of PLD compared with PLC, it is now clear that, like PLC, PLD does not exist as a single protein, but represents a family of different isozymes. The first evidence that multiple PLD isozymes existed in mammalian cells came from fractionation studies on HL60 cells and porcine brain membranes that identified at least two separate PLD molecules with different regulatory properties[148,169]. The first enzyme displayed a molecular mass of about 200 kDa and was activated by sodium oleate but was insensitive to Arf. The second enzyme had a molecular mass of about 95 kDa, and was sensitive to Arf but not to sodium oleate.

The cloning of a PLD molecule was reported in 1995[170]. This enzyme, named PLD1, is a 120 kDa protein present in cells as two variant forms derived from alternative splicing[159]. Because PLD1 requires PIP_2 as a cofactor for enzymatic activity and is insensitive to sodium oleate, it therefore probably corresponds to the previously described 95 kDa form from porcine brain. The PLD1 molecule does not possess domains such as C2, SH2, or SH3 that frequently are found in other signalling proteins. A PH-like domain is present in the N-terminal portion of the protein, and it has been initially supposed to be involved in PIP_2 enzyme regulation. However, a recent piece of work demonstrates that although PLD1 directly binds to PIP_2, this interaction does not involve the PH domain[171]. PLD1 has a low basal activity, that is at least partially caused by the inhibitory effect of a sequence that is exclusive of this isoform and indicated as the 'loop region', located in the center of the protein[172]. Enzymatic activity of PLD1 can be strongly stimulated by PKCα and by members of the Arf and Rho family of small G-proteins. Interestingly, these modulators act synergistically in activating PLD1, suggesting that they bind to different regions of the protein[172]. Deletion of the N-terminal third of PLD1 attenuates regulation by PKC but not by other activators, and deletion of the C-terminal third reduces activation by RhoA. The site of interaction of PLD1 with Arf has not been identified.

A second PLD isozyme, called PLD2, was cloned in 1997[173]. PLD2 is approximately 50% identical to PLD1, but has a molecular mass slightly smaller. As PLD1, PLD2 requires PIP_2 as a cofactor for enzymatic activity. PLD2 has a different subcellular localization than PLD1 being predominantly localized at the plasma membrane in cells, whereas PLD1 is found exclusively in the perinuclear region in association with the Golgi complex and the endoplasmatic reticulum[174]. Cloned PLD2 exhibits a very high basal activity, about 1500-fold greater than that of PLD1[173]. Studies with deletion mutants demonstrated that the N-terminal portion of PLD2 is necessary to confer this high basal activity to the enzyme[174]. Moreover, in contrast to PLD1, the basal activity of PLD2 cannot be further stimulated in vitro by PKCα, Arf, or Rho proteins[173]. Because such

Cambridge Books Online © Cambridge University Press, 2009

a high PLD activity is not detected in lysate of cells naturally expressing PLD2, it has been proposed that this isoform is constitutively inhibited in vivo, and that its activation is achieved by removal of such inhibition. An 18 kDa protein that is not a small GTPase, has been identified as a possible PLD2 inhibitor. This protein is still to be further characterized[173]. More recently, it has been shown that, upon deletion of the N-terminal portion, PLD2 not only loses most of its typical high basal activity, but also becomes responsive to Arf, but not to Rho or PKC[174]. These results suggest that PLD2 may be regulated in vivo by proteolysis into an Arf-responsive isoform.

Activation and regulation of phospholipase D in platelets

Compared with PLC, very little is known about PLD activation in platelets. No more than 20 papers have documented PLD activation in agonist-stimulated platelets by means of measuring formation of PA, choline, or products of the transphosphatidylation reaction. Moreover, no reports describe the expression of the cloned PLD isozymes in human platelets. Despite this, it is now clear that one or more PLD molecules exist in these cells and can be activated upon stimulation with different agonists. The first evidence for the presence of an agonist-activated PLD in platelets was reported in 1988. Using ^{3}H-arachidonic acid-labelled human platelets, it was shown that, in the presence of ethanol, thrombin induced the accumulation of ^{3}H phosphatidylethanol in a time- and dose-dependent manner[175]. Activation of PLD was also deduced from the observed accumulation of ^{3}H-choline and ^{3}H-phosphocholine in labelled platelets stimulated with thrombin or collagen[146]. By contrast, no release of these labelled metabolites was observed in response to ADP, PAF or epinephrine. However, because a PC-PLC is present in platelets, the real contribution of PLD in the generation of labelled choline and phosphocholine is difficult to assess. Stimulation of PLD in human platelets has also been described in response to the TxA_2 analogue U46619[176]. Finally, measurement of the transphosphatidylation reaction in platelet membrane or permeabilized cells demonstrates that PLD can be activated in these cells upon treatment with GTP, GTPγS, calcium ionophore A23187, and phorbol myristate acetate (PMA)[176–178].

The biochemical mechanism for PLD activation in platelets clearly involves multiple signalling pathways. In thrombin and collagen-stimulated platelets, PLD activation is not affected by aspirin, indicating that it is a primary event that does not require the action of endogenous TxA_2[146,179]. However, recent work showed that the ADP scavenger apyrase and the purinergic receptor antagonist ARL 66096 totally block PLD activation induced by low concentrations of thrombin and strongly reduce that promoted by high concentrations of this agonist[180]. In addition, ADP scavengers or antagonists strongly inhibit PLD activation stimulated by TxA_2. Although ADP alone is not able to stimulate PLD activity, this agent can reinforce the effects of low doses of stimulatory agonists like thrombin[180]. This indicates that secreted ADP may provide an amplification mechanism for agonist-induced PLD activation. Several studies indicate that activation of PLD can be mediated by an increase in the intracellular concentration of Ca^{2+}. This is based on studies using permeabilized platelets or intact cells treated with the Ca^{2+} ionophore A23187[176,178,181]. Moreover, chelation of intracellular Ca^{2+} by BAPTA prevents PLD activation[181,182]. In addition, production of phosphatidylethanol can be stimulated by PMA and can be blocked by inhibitors of PKC[176,177], suggesting a role for PKC in the stimulation of platelet PLD.

Finally, studies on platelet membrane and on permeabilized platelets clearly show a stimulatory effect of GTP and GTPγS on PLD activity[177,181,183], indicating the involvement of GTP-binding proteins. Therefore, G-proteins, PKC, and Ca^{2+} may act as activators of PLD in platelets. However, their actions may not be direct, but indicate that PLD stimulation in platelets is secondary to activation of PLC.

Still controversial is the regulation of PLD by integrin $\alpha_{IIb}\beta_3$ and platelet aggregation. Some authors report that, in thrombin- and collagen-stimulated platelets, PLD activation occurs in parallel with aggregation and can be blocked by chelation of extracellular calcium or by the omission of stirring[146,179]. Moreover, high-density lipoproteins (HDL_3), which are proposed to bind to the integrin receptor, have been shown to induce activation of PLD[184]. In the presence of butanol, accumulation of phosphatidylbutanol induced by HDL_3 was not observed in platelets from Glanzmann's thrombasthenia patients lacking integrin $\alpha_{IIb}\beta_3$[184]. By contrast, a different work reported that thrombin and PMA were still able to stimulate PLD in thrombasthenic platelets as well as in normal platelets in which fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ was prevented by the antagonistic peptide RGDS[176]. Therefore, it appears that two mechanisms for PLD activation, one dependent, and one independent of integrin $\alpha_{IIb}\beta_3$, exist in platelets.

Finally, a role for protein tyrosine-kinases in activation of platelet PLD has been proposed[185]. Pervanadate, an inhibitor of protein-tyrosine phosphatases, stimulates PLD activity, and some inhibitors of tyrosine kinases, such as genistein and tyrphostins, markedly reduce PLD activation

Cambridge Books Online © Cambridge University Press, 2009

induced by thrombin in intact platelets and by GTPγS in permeabilized platelets[185].

Although no doubt exists that PLD is activated in agonist-stimulated platelets, the role of this event in cell activation is still controversial. Several studies suggest that PLD may actually contribute to PA formation induced by platelet stimulation, but this contribution may not be significant. It has been calculated that upon stimulation with thrombin, only 13% of incremental PA arises from PLD activation, and most can be ascribed to the combined action of PLC on PIP_2 and DAG kinase[178,182]. These results seem to limit the physiological importance of PLD in platelets. PA generated by the action of PLD can be converted to DAG by a PA-phosphohydrolase. It was reported that accumulation of DAG in thrombin-stimulated platelets is actually biphasic[186]. The first phase of DAG production is rapid and transient, peaks at 10 seconds, and is concomitant with IP_3 production. Therefore, it is most likely that it derives exclusively from the action of PLC on PIP_2. The second phase of DAG production is more sustained, but delayed, peaking at 2–3 minutes when the concentration of IP_3 has returned to the basal levels. Moreover, in thrombin-stimulated platelets a decrease of membrane PC was measured. However, it was found that the decrease of PC was mainly caused by the action of PLA_2 rather than PLD, and that the delayed accumulation of DAG probably derived from hydrolysis of PI by PLC rather than by hydrolysis of PC by PLD[186]. Again, this study limits the possible physiological significance of PLD in platelets. By contrast, other authors report evidence suggesting that PLD may be involved in the regulation of platelet secretion. This is based on studies with permeabilized platelets showing that secretion correlated with PLD, but not with PLC activation, and suggesting that PLD may mediate this platelet response by PKC-dependent and PKC-independent mechanisms[182]. A role for PLD in regulating platelet secretion and aggregation was also suggested based on the finding that addition of PA resulted in shape change and reversible aggregation[179]. Moreover, PA was shown to shorten the latency period of platelet aggregation induced by collagen[179]. Despite these observations, the real effect of PLD activation in platelet response to extracellular agonists remains largely unknown.

Conclusions

This chapter describes the considerable progress made over the last three decades in the understanding of the roles of PLC and PLD in the biology of platelet responses. These phospholipases are critical for the formation of numerous second messengers, including inositol trisphosphate, diacylglycerol, phosphatidic acid, lysophosphatidic acid, calcium release and activation of protein kinases. Our increased knowledge of these signalling molecules has introduced the potential for therapeutic intervention in conditions of altered platelet activity.

References

1 Rebecchi MJ, Pentyala SN. Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiol Rev* 2000; 80: 1291–1335.
2 Rittenhouse SE. Phosphoinositide 3-kinase activation and platelet function. *Blood* 1996; 88: 4401–4414.
3 Fox JEB. The platelet cytoskeleton. *Thromb Haemost* 1993; 70: 884–893.
4 Luckhoff A, Clapham DE. Inositol 1,3,4,5-tetrakisphosphate activates an endothelial Ca^{2+}-permeable channel. *Nature* 1992; 355: 356–358.
5 Cullen PJ, Hsuan JJ, Truong O, Letcher AJ, Jackson TR, Dawson, AP, Irvine RF. Identification of a specific Ins(1,3,4,5)P_4-binding protein as a member of the GAP1 family. *Nature* 1995; 376: 527–530.
6 Torti M, Lapetina EG. Structure and function of rap proteins in human platelets. *Thromb Haemost* 1994; 71: 533–543.
7 Nozawa Y, Nakashima S, Nagata K. Phospholipid-mediated signalling in receptor activation of human platelets. *Biochim Biophys Acta* 1991; 1082: 219–238.
8 Kroll MH, Zavoico GB, Schafer AI. Second messenger function of phosphatidic acid in platelet activation. *J Cell Physiol* 1989; 139: 558–564.
9 Jalink K, Hordijk PL, Moolenaar WH. Growth factor-like effects of lysophosphatidic acid, a novel lipid mediator. *Biochim Biophys Acta* 1994; 1198: 185–196.
10 Kroll MH, Schafer AI. Biochemical mechanisms of platelet activation. *Blood* 1989; 74: 1181–1196.
11 Siess W, Weber PC, Lapetina EG. Activation of phospholipase C is dissociated from arachidonate metabolism during platelet shape change induced by thrombin or platelet-activating factor. Epinephrine does not induce phospholipase C activation or platelet shape change. *J Biol Chem* 1984; 259: 8286–8292.
12 Banga HS, Simons ER, Brass LF, Rittenhouse SE. Activation of phospholipases A and C in human platelets exposed to epinephrine: role of glycoprotein IIb/IIIa and dual role of epinephrine. *Proc Natl Acad Sci USA* 1986; 83: 9197–9201.
13 Vickers JD, Kinlough-Rathbone RL, Packham MA, Mustard JF. Inositol phospholipid metabolism in human platelets stimulated by ADP. *Eur J Biochem* 1990; 193: 521–528.
14 Vickers JD. ADP-stimulated fibrinogen binding is necessary for some of the inositol phospholipid changes found in ADP-stimulated platelets. *Eur J Biochem* 1993; 216: 231–237.

Cambridge Books Online © Cambridge University Press, 2009

15 Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB, Kunapuli SP. Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. *J Biol Chem* 1998; 273: 2024–2029.
16 Jin J, Daniel JL, Kunapuli SP. Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J Biol Chem* 1998; 273: 2030–2034.
17 Baldassare JJ, Fisher GJ. Regulation of membrane-associated and cytosolic phospholipase C activities in human platelets by guanosine triphosphate. *J Biol Chem* 1986; 261: 11942–11944.
18 Haslam RJ, Davidson MML. Receptor-induced diacylglycerol formation in permeabilized platelets: possible role for a GTP binding protein. *J Recept Res* 1984; 4: 605–629.
19 Culty M, Davidson MM, Haslam RJ. Effects of guanosine 5'-[γ-tio]triphosphate and thrombin on the phosphoinositide metabolism of electropermeabilized human platelets. *Eur J Biochem* 1988; 171: 523–533.
20 Siess W, Cuatrecasas P, Lapetina EG. A role for cyclooxygenase products in the formation of phosphatidic acid in stimulated human platelets. Differential mechanisms of action of thrombin and collagen. *J Biol Chem* 1983; 258: 4683–4686.
21 Kroll MH, Harris TS, Moake JL, Handin RI, Schafer AI. Von Willebrand factor binding to platelet GPIb initiates signals for platelet activation. *J Clin Invest* 1991; 88: 1568–1573.
22 Yatomi Y, Ozaki Y, Koike Y, Satoh K, Kume S. Wheat germ agglutinin-induced intracellular calcium mobilization in human platelets: suppression by staurosporine and resistance to cyclic AMP inhibition. *Biochem Biophys Res Commun* 1993; 19: 453–458.
23 Torti M, Balduini C, Ramaschi G, Sinigaglia F. Stimulation of human platelets with concanavalin A involves phospholipase C activation. *Cell Biochem Funct* 1992; 10: 53–59.
24 Anderson GP, Anderson CL. Signal transduction by the platelet Fc receptor. *Blood* 1990; 76: 1165–1172.
25 Gironcel D, Racaud-Sultan C, Payrastre B et al. $\alpha_{IIb}\beta_3$-integrin mediated adhesion of human platelets to a fibrinogen matrix triggers phospholipase C activation and phosphatidylinositol 3′,4′-bisphosphate accumulation. *FEBS Lett* 1996; 389: 253–256.
26 Sinigaglia F, Torti M, Ramaschi G, Balduini C. The occupancy of glycoprotein IIb-IIIa complex modulates thrombin activation of human platelets. *Biochim Biophys Acta* 1989; 984: 225–230.
27 Yamaguchi A, Tanoue K, Yamazaki H. Secondary signals mediated by GPIIb/IIIa in thrombin-activated platelets. *Biochim Biophys Acta* 1990; 1054: 8–13.
28 Chau L, Tai H. Resolution into two different forms and study of the properties of phosphatidylinositol-specific phospholipase C from human platelet cytosol. *Biochim Biophys Acta* 1982; 713: 344–351.
29 Banno Y, Yada Y, Nozawa Y. Purification and characterization of membrane-bound phospholipase C specific for phosphoinositides from human platelets. *J Biol Chem* 1988; 263: 11459–11465.
30 Manne V, Kung H. Characterization of phosphoinositide-specific phospholipase C from human platelets. *Biochem J* 1987; 243: 763–771.
31 Nakashima S, Banno Y, Nozawa Y. Platelet phospholipase C and D. In von Bruchhausen F, Walter U, *Handbook of experimental pharmacology, vol 126: Platelets and their factors*. Berlin, Heidelberg, New York: Springer-Verlag: 1997; 231–246.
32 Rhee SG, Suh P, Ryu S, Lee SY. Studies of inositol phospholipid-specific phospholipase C. *Science* 1989; 244: 546–550.
33 Torti M, Lapetina EG. Role of rap1b and p21rasGTPase activating protein in the regulation of phospholipase C-γ1 in human platelets. *Proc Natl Acad Sci USA* 1992; 89: 7796–7800.
34 Banno Y, Yu A, Nakashima T, Homma Y, Takenawa T, Nozawa Y. Purification and characterization of a cytosolic phosphoinositide-phospholipase C (γ2-type) from human platelets. *Biochem Biophys Res Commun* 1990; 167: 369–401.
35 Lee SB, Rao AK, Lee K, Yang X, Bae YS, Rhee SG. Decreased expression of phospholipase C-β2 isozyme in human platelets with impaired function. *Blood* 1996; 88: 1684–1691.
36 Pawelczyk T, Matecki A. Localization of phospholipase Cδ3 in the cell and regulation of its activity by phospholipids and calcium. *Eur J Biochem* 1998; 257: 169–177.
37 Taylor SJ, Chae HZ, Rhee SG, Exton JH. Activation of the β1 isozyme of phospholipase C by α subunits of the Gq class of G proteins. *Nature* 1991; 350: 516–518.
38 Lee CH, Park D, Wu D, Rhee SG, Simon MI. Members of the Gq α subunit gene family activate phospholipase C β isozymes. *J Biol Chem* 1992; 267: 16044–16047.
39 Shenker A. Goldsmith P, Unson CG, Spiegel AM. The G protein coupled to the thromboxane A_2 receptor in human platelets is a member of the novel Gq family. *J Biol Chem* 1991; 266: 9309–9313.
40 Van Willigen G, Donath J, Lapetina EG, Akkerman JW. Identification of α-subunits of trimeric GTP-binding proteins in human platelets by RT-PCR. *Biochem Biophys Res Commun* 1995; 214: 254–262.
41 Park D, Jhon D, Kriz R, Knopf J, Rhee SG. Cloning, sequencing, expression, and Gq-independent activation of phospholipase C-β2. *J Biol Chem* 1992; 267: 16048–16055.
42 Jhon D, Lee H, Park D, Lee C, Lee K, Yoo OJ, Rhee SG. Cloning, sequencing, purification, and Gq-dependent activation of phospholipase C-β3. *J Biol Chem* 1993; 268: 6654–6661.
43 Camps M, Hou C, Sidiropoulos D, Stock JB, Jakobs KH, Gierschik P. Stimulation of phospholipase C by guanine-nucleotide-binding protein $\beta\gamma$ subunits. *Eur J Biochem* 1992; 206: 821–831.
44 Blank JL, Brattain KA, Exton JH. Activation of cytosolic phosphoinositide phospholipase C by G-protein $\beta\gamma$ subunits. *J Biol Chem* 1992; 267: 23069–23075.
45 Camps M, Carozzi A, Schnabel P, Scheer A, Parker PJ, Gierschik P. Isozyme-selective stimulation of phospholipase C-β2 by G protein $\beta\gamma$-subunits. *Nature* 1992; 360: 684–686.
46 Katz A, Wu D, Simon MI. Subunits $\beta\gamma$ of heterotrimeric G protein activate β2 isoform of phospholipase C. *Nature* 1992; 360: 686–689.

Cambridge Books Online © Cambridge University Press, 2009

47 Park D, Jhon D, Lee C, Lee K, Rhee SG. Activation of phospholipase C isozymes by G protein $\beta\gamma$ subunits. *J Biol Chem* 1993; 268: 4573–4576.

48 Carpenter G, Ji Qs. Phospholipase C-γ as a signal-transducing element. *Exp Cell Res* 1999; 253: 15–24.

49 Vassbotn FS, Havnen OK, Heldin CH, Holmsen H. Negative feedback regulation of human platelets via autocrine activation of the platelet-derived growth factor α-receptor. *J Biol Chem* 1994; 269: 13874–13879.

50 Allen V, Swigart P, Cheung R, Cockcroft S, Katan M. Regulation of inositol lipid-specific phospholipase C-δ by changes in Ca^{2+} ion concentrations. *Biochem J* 1997; 327: 545–552.

51 Feng JF, Rhee SG, Im MJ. Evidence that phospholipase Cδ is the effector in the Gh (transglutaminase II)-mediated signalling. *J Biol Chem* 1996; 271: 16451–16454.

52 Homma Y, Emori Y. A dual functional signal mediator showing RhoGAP and phospholipase C-δ stimulating activities. *EMBO J* 1995; 14: 286–291.

53 Torti M, Lapetina EG. The role of low molecular weight GTP-binding proteins in human platelets. In: Lapetina EG *Advances in molecular and cell biology: the platelet*, Greenwich CT: JAI Press; 1997: 197–251.

54 Vezza R, Habib A, FitzGerald GA. Differential signalling by the thromboxane receptor isoforms via the novel GTP-binding protein Gh. *J Biol Chem* 1999; 274: 12774–12779.

55 Essen L, Perisic O, Cheung R, Katan M, Williams RL. Crystal structure of a mammalian phosphoinositide-specific phospholipase Cδ. *Nature* 1996; 380: 595–602.

56 Essen L, Perisic O, Katan M, Wu Y, Roberts MF, Williams RL. Structural mapping of the catalytic mechanism for a mammalian phosphoinositide-specific phospholipase C. *Biochemistry* 1997; 36: 1704–1718.

57 Serunian LA, Haber MT, Fukui T et al. Polyphosphoinositides produced by phosphatidylinositol 3-kinase are poor substrates for phospholipase C from rat liver and bovine brain. *J Biol Chem* 1989; 264: 17809–17815.

58 Ellis MV, James SR, Perisic O, Downes P, Williams RL, Katan M. Catalytic domain of phosphoinositide-specific phospholipase C (PLC). *J Biol Chem 1998*; 273: 11650–11659.

59 Smith MR, Liu YL, Matthews NT, Rhee SG, Sung WK, Kung HF. Phospholipase C-γ1 can induce DNA synthesis by a mechanism independent of its lipase activity. *Proc Natl Acad Sci USA* 1994; 91: 6554–6558.

60 Lemmon MA, Ferguson KM. Signal-dependent membrane targeting by pleckstrin homology (PH) domains. *Biochem J* 2000; 350: 1–18.

61 Abrams CS, Wu H, Zhao W, Belmonte E, White D, Brass LF. Pleckstrin inhibits phosphoinositide hydrolysis initiated by G-protein-coupled and growth factor receptors. A role for pleckstrin's PH domains. *J Biol Chem* 1995; 270: 14485–14492.

62 Lemmon MA, Ferguson KM, O'Brien R, Sigler PB, Schlessinger J. Specific and high-affinity binding of inositol phosphates to an isolated pleckstrin homology domain. *Proc Natl Acad Sci USA* 1995; 92: 10472–10476.

63 Garcia P, Gupta R, Shah S et al. The pleckstrin homology domain of phospholipase C-δ1 binds with high affinity to phosphatidylinositol 4,5-bisphosphate in bilayer membranes. *Biochemistry* 1995; 34: 16228–16234.

64 Ferguson KM, Lemmon MA, Schlessinger J, Sigler PB. Structure of the high affinity complex of inositol trisphosphate with a phospholipase C pleckstrin homology domain. *Cell* 1995; 83: 1037–1046.

65 Falasca M, Logan SK, Lehto VP, Baccante G, Lemmon MA, Schlessinger J. Activation of phospholipase Cγ by PI 3-kinase-induced PH domain-mediated membrane targeting. *EMBO J* 1998; 17: 414–422.

66 Bae YS, Cantley LG, Chen CS, Kim S, Kwon KS, Rhee SG. Activation of phospholipase C-γ by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 1998; 273: 4465–4469.

67 Rameh LE, Rhee SG, Spokes K, Kazlauskas A, Cantley LC, Cantley LG. Phosphoinositide 3-kinase regulates phospholipase Cγ-mediated calcium signalling. *J Biol Chem* 1998; 273: 23750–23757.

68 Pasquet J, Bobe R, Gross B et al. A collagen-related peptide regulates phospholipase Cγ2 via phosphatidylinositol 3-kinase in human platelets. *Biochem J* 1999; 342: 171–177.

69 Gratacap M, Payrastre B, Viala C, Mauco G, Plantavid M, Chap H. Phosphatidylinositol 3,4,5-trisphosphate-dependent stimulation of phospholipase Cγ2 is an early key event in FcγRIIA-mediated activation of human platelets. *J Biol Chem* 1998; 273: 24314–24321.

70 Tall E, Dorman G, Garcia P et al. Phosphoinositide binding specificity among phospholipase C isozymes as determined by photo-cross-linking to novel substrate and product analogs. *Biochemistry* 1997; 36: 7239–7248.

71 Wang T, Pentyala S, Rebecchi MJ, Scarlata S. Differential association of the pleckstrin homology domains of phospholipases C-β1, C-β2 and C-δ1 with lipid bilayers and the $\beta\gamma$ subunits of heterotrimeric G proteins. *Biochemistry* 1999; 38: 1517–1524.

72 Touhara K, Inglese J, Pitcher JA, Shaw G, Lefkowitz RJ. Binding of G protein $\beta\gamma$ subunits to pleckstrin homology domains. *J Biol Chem* 1994; 269: 10217–10220.

73 Romoser V, Ball R, Smrcka AV. Phospholipase Cβ2 association with phospholipid interfaces assessed by fluorescence resonance energy transfer. *J Biol Chem* 1996; 271: 25071–25078.

74 Wang T, Dowal L, El-Maghrabi MR, Rebecchi M, Scarlata S. The pleckstrin homology domain of phospholipase C β2 links the binding of G $\beta\gamma$ to activation of the catalytic core. *J Biol Chem* 2000; 275: 7466–7469.

75 Razzini G, Brancaccio A, Lemmon MA, Guarnier S, Falasca M. The role of the pleckstrin homology domain in membrane targeting and activation of phospholipase Cβ1. *J Biol Chem* 2000; 275: 14873–14881.

76 Kuang Y, Wu Y, Smrcka A, Jiang H, Wu D. Identification of a phospholipase Cβ2 region that interacts with G$\beta\gamma$. *Proc Natl Acad Sci USA* 1996; 93: 2964–2968.

77 Grobler JA, Hurley JH. Catalysis by phospholipase Cδ1 requires that Ca^{2+} binds to the catalytic domain, but not to the C2 domain. *Biochemistry* 1998; 37: 5020–5028.

Cambridge Books Online © Cambridge University Press, 2009

78 Nakashima S, Banno Y, Watanabe T et al. Deletion and site-directed mutagenesis of EF-hand domain of phospholipase C-δ1: effects on its activity. *Biochem Biophys Res Commun* 1995; 211: 364–369.
79 Rizo J, Sudhof TC. C2-domains, structure and function of a universal Ca^{2+}-binding domain. *J Biol Chem* 1998; 273: 15879–15882.
80 Gross BS, Melford SK, Watson SP. Evidence that phospholipase C-γ2 interacts with SLP-76, Syk, Lyn, LAT and the Fc receptor γ-chain after stimulation of the collagen receptor glycoprotein VI in human platelets. *Eur J Biochem* 1999; 263: 612–623.
81 Bar-Sagi D, Rotin D, Batzer A, Mandiyan V, Schlessinger J. SH3 domains direct cellular localization of signalling molecules. *Cell* 1993; 74: 83–91.
82 Ellis MV, Carne A, Katan M. Structural requirements of phosphatidylinositol-specific phospholipase Cδ1 for enzyme activity. *Eur J Biochem* 1993; 213: 339–347.
83 Sankaran B, Osterhout J, Wu D, Smrcka AV. Identification of a structural element in phospholipase Cβ2 that interacts with G protein $\beta\gamma$ subunits. *J Biol Chem* 1998; 273: 7148–7154.
84 Wu D, Jiang H, Katz A, Simon MI. Identification of critical regions on phospholipase C-β1 required for activation by G-proteins. *J Biol Chem* 1993; 268: 3704–3709.
85 Park D, Jhon DY, Lee CW Ryu SH, Rhee SG. Removal of the carboxyl-terminal region of phospholipase Cβ1 by calpain abolishes activation by Gαq. *J Biol Chem* 1993; 268; 3710–3714.
86 Lee SB, Shin SH Hepler JR, Gilman AG, Rhee SG. Activation of phospholipase C-β2 mutants by G protein αq and $\beta\gamma$ subunits. *J Biol Chem* 1993; 268: 25952–25957.
87 Paulssen RH, Woodson J, Liu Z, Ross EM. Carboxyl-terminal fragments of phospholipase C- β1 with intrinsic Gq GTPase-activating protein (GAP) activity. *J Biol Chem* 1996; 271: 26622–26629.
88 Mao GF, Kunapuli SP, Rao AK. Evidence for two alternatively spliced forms of phospholipase C-β2 in haematopoietic cells. *Brit J Haematol* 2000; 110: 402–408.
89 Knezevic I, Borg C, Le Breton GC. Identification of Gq as one of the G-proteins which copurify with human platelet thromboxane A_2/prostaglandin H_2 receptors. *J Biol Chem* 1993; 268: 26011–26017.
90 Baldassare JJ, Tarver AP, Henderson PA, Mackin WM, Sahagan B, Fisher GJ. Reconstitution of thromboxane A_2 receptor-stimulated phosphoinositide hydrolysis in isolated platelet membranes: involvement of phosphoinositide-specific phospholipase C-β and GTP-binding protein Gq. *Biochem J* 1993; 291: 235–240.
91 Amatruda III TT, Steele DA, Slepak VZ, Simon MI. Gα16, a G protein α subunit specifically expressed in hematopoietic cells. *Proc Natl Acad Sci USA* 1991; 88: 5587–5591.
92 Brass LF, Shaller CC, Belmonte EJ. Inositol 1,4,5-triphosphate-induced granule secretion in platelets. Evidence that the activation of phospholipase C mediated by platelet thromboxane receptors involves a guanine nucleotide binding protein-dependent mechanism distinct from that of thrombin. *J Clin Invest* 1987; 79: 1269–1275.
93 Offermanns S, Toombs CF, Hu Y, Simon MI. Defective platelet activation in Gαq-deficient mice. *Nature* 1997; 389: 183–186.
94 Offermanns S, Laugwitz K, Spicher K, Schultz G. G proteins of the G12 family are activated via thromboxane A_2 and thrombin receptors in human platelets. *Proc Natl Acad Sci USA* 1994; 91: 504–508.
95 Brass LF, Laposata M, Banga HS, Rittenhouse SE. Regulation of the phosphoinositide hydrolysis pathway in thrombin-stimulated platelets by a pertussis toxin-sensitive guanine nucleotide-binding protein. Evaluation of its contribution to platelet activation and comparisons with the adenylate cyclase inhibitory protein, Gi. *J Biol Chem* 1986; 261: 16838–16847.
96 Grandt R, Aktories K, Jakobs KH. Evidence for two GTPases activated by thrombin in membranes of human platelets. *Biochem J* 1986; 237: 669–674.
97 Benka ML, Lee M, Wang G et al. The thrombin receptor in human platelets is coupled to a GTP binding protein of the Gαq family. *FEBS Lett* 1995; 363: 49–52.
98 Ohlmann P, Laugwitz K, Nurnberg B, Spicher K, Schultz G, Cazenave J, Gachet C. The human platelet ADP receptor activates Gi2 proteins. *Biochem J* 1995; 312: 775–779.
99 Banno Y, Nakashima S, Hachiya T, Nozawa Y. Endogenous cleavage of phospholipase C-β3 by agonist-induced activation of calpain in human platelets. *J Biol Chem* 1995; 270: 4318–4324.
100 Banno Y, Asano T, Nozawa Y. Proteolytic modification of membrane-associated phospholipase C-β by μ-calpain enhances its activation by G-protein $\beta\gamma$ subunits in human platelets. *FEBS Lett* 1994; 340: 185–188.
101 Nozawa Y, Banno Y, Nagata K. Regulation of phosphoinositide-specific phospholipase C activity in human platelets. *Adv Exp Med Biol* 1993; 344: 37–47.
102 Blake RA, Schieven GL, Watson SP. Collagen stimulates tyrosine phosphorylation of phospholipase C-γ2 but not phospholipase C-γ1 in human platelets. *FEBS Lett* 1994; 353: 212–216.
103 Guinebault C, Payrastre B, Sultan C, Mauco G, Breton M, Levy-Toledano S, Plantavid M, Chap H. Tyrosine kinases and phosphoinositide metabolism in thrombin-stimulated human platelets. *Biochem J* 1993; 292: 851–856.
104 Sekiya F, Bae YS, Rhee SG. Regulation of phospholipase C isozymes: activation of phospholipase C-γ in the absence of tyrosine-phosphorylation. *Chem Phys Lipids* 1999; 98: 3–11.
105 Tate BF, Rittenhouse SE. Thrombin activation of human platelets causes tyrosine phosphorylation of PLC-γ2. *Biochim Biophys Acta* 1993; 1178: 281–285.
106 Daniel JL, Dangelmaier C, Smith JB. Evidence for a role for tyrosine phosphorylation of phospholipase Cγ2 in collagen-induced platelet cytosolic calcium mobilization. *Biochem J* 1994; 302: 617–622.
107 Torti M, Bertoni A, Canobbio I, Sinigaglia F, Lapetina EG, Balduini C. Rap1b and rap2b translocation to the cytoskele-

Cambridge Books Online © Cambridge University Press, 2009

ton by von Willebrand factor involves FcγII receptor-mediated protein tyrosine phosphorylation. *J Biol Chem* 1999; 274: 13690–13697.
108 Torti M, Tolnai Festetics E, Bertoni A, Sinigaglia F, Balduini C. Clustering of integrin $\alpha_{IIb}\beta_3$ differently regulates tyrosine phosphorylation of pp72^{syk}, PLCγ2 and pp125FAK in concanavalin A-stimulated platelets. *Thromb Haemost* 1999; 81: 124–130.
109 Clemetson JM, Polgar J, Magnenat E, Wells TNC, Clemetson KJ. The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcαR and the natural killer receptors. *J Biol Chem* 1999; 274: 29019–29024.
110 Jandrot-Perrus M, Busfield S, Lagrue A et al. Cloning, characterization, and functional studies of human and mouse glycoprotein VI: a platelet-specific collagen receptor from the immunoglobulin superfamily. *Blood* 2000; 96: 1798–1807.
111 Tsuji M, Ezumi Y, Arai M, Takayama H. A novel association of Fc receptor γ-chain with glycoprotein VI and their co-expression as a collagen receptor in human platelets. *J Biol Chem* 1997; 272: 23528–23531.
112 Ichinohe T, Takayama H, Ezumi Y et al. Collagen-stimulated activation of Syk but not c-Src is severely compromised in human platelets lacking membrane glycoprotein VI. *J Biol Chem* 1997; 272: 63–68.
113 Ichinohe T, Takayama H, Ezumi Y, Yanagi S, Yamamura H, Okuma M. Cyclic AMP-insensitive activation of c-Src and Syk protein-tyrosine kinases through platelet membrane glycoprotein VI. *J Biol Chem* 1995; 270: 28029–28036.
114 Asselin J, Gibbins JM, Achison M et al. A collagen-like peptide stimulates tyrosine phosphorylation of Syk and phospholipase Cγ2 in platelets independent of integrin $\alpha_2\beta_1$. *Blood* 1997; 89: 1235–1242.
115 Francischetti IM, Ghazaleh FA, Reis RA, Carlini CR, Guimaraes JA. Convulxin induces platelet activation by a tyrosine-kinase-dependent pathway and stimulates tyrosine phosphorylation of platelet proteins, including PLCγ2, independently of integrin $\alpha_{IIb}\beta_3$. *Arch Biochem Biophys* 1998; 353: 239–250.
116 Keely PJ, Parise LV. The $\alpha_2\beta_1$ integrin is a necessary co-receptor for collagen-induced activation of Syk and the subsequent phosphorylation of phospholipase Cγ2 in platelets. *J Biol Chem* 1996; 271: 26668–26676.
117 Poole A, Gibbins JM, Turner M et al. The Fc receptor γ-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *EMBO J* 1997; 16: 2333–2341.
118 Gibbins J, Asselin J, Farndale R, Barnes M, Law CL, Watson SP. Tyrosine phosphorylation of the Fc receptor γ-chain in collagen stimulated platelets. *J Biol Chem* 1996; 271: 18095–18099.
119 Quek LS, Pasquet J, Hers I et al. Fyn and Lyn phosphorylate the Fc receptor γ-chain downstream of glycoprotein VI in murine platelets, and Lyn regulates a novel feedback pathway. *Blood* 2000; 96: 4246–4253.
120 Chacko GW, Brandt JT, Coggeshall KM, Anderson CL. Phosphoinositide 3-kinase and p72syk noncovalently associate with the low affinity Fcγ receptor on human platelets through an immunoreceptor tyrosine-based activation motif. Reconstitution with synthetic phosphopeptides. *J Biol Chem* 1996; 271: 10775–10781.
121 Falati S, Edmead CE, Poole AW. Glycoprotein Ib-V-IX, a receptor for von Willebrand factor, couples physically and functionally to the Fc receptor γ-chain, Fyn, and Lyn to activate human platelets. *Blood* 1999; 94: 1648–1656.
122 Quek LS, Bolen J, Watson SP. A role for Bruton's tyrosine kinase (Btk) in platelet activation by collagen. *Curr Biol* 1998; 8: 1137–1140.
123 Gross BS, Lee JR, Clements JL et al. Tyrosine phosphorylation of SLP-76 is downstream of Syk following stimulation of the collagen receptor in platelets. *J Biol Chem* 1999; 274: 5963–5971.
124 Clements JL, Lee JR, Gross B et al. Fetal hemorrhage and platelet dysfunction in SLP-76-deficient mice. *J Clin Invest* 1999; 103: 19–25.
125 Pasquet J, Gross B, Quek L et al. LAT is required for tyrosine phosphorylation of phospholipase Cγ2 and platelet activation by the collagen receptor GPVI. *Mol Cell Biol* 1999; 19: 8326–8334.
126 Bobe R, Wilde JI, Maschberger P et al. Phosphatidylinositol 3-kinase-dependent translocation of phospholipase Cγ2 in mouse megakaryocytes is independent of Bruton tyrosine kinase translocation. *Blood* 2001; 97: 678–684.
127 Ritthenhouse SE, Allen CL. Synergistic activation by collagen and 15-hydroxy-9α,11α-peroxidoprosta-5,13-dienoic acid (PGH2) of phosphatidylinositol metabolism and arachidonic acid release in human platelets. *J Clin Invest* 1982; 70: 1216–1224.
128 Nakano T, Hanasaki K, Arita H. Possible involvement of cytoskeleton in collagen-stimulated activation of phospholipases in human platelets. *J Biol Chem 1989*; 264: 5400–5406.
129 Pignatelli P, Pulcinelli FM, Lenti L, Gazzaniga PP, Violi F. Hydrogen peroxide is involved in collagen-induced platelet activation. *Blood* 1998; 94: 484–490.
130 Hers I, Berlanga O, Tiekstra MJ, Kamiguti AS, Theakston RD, Watson SP. Evidence against a direct role of the integrin $\alpha_2\beta_1$ in collagen-induced tyrosine phosphorylation in human platelets. *Eur J Biochem* 2000; 267: 2088–2097.
131 Inoue K, Ozaki Y, Satoh K et al. Signal transduction pathways mediated by glycoprotein Ia/IIa in human platelets: comparison with those of glycoprotein VI. *Biochem Biophys Res Commun* 1999; 256: 114–120.
132 Kamiguti AS, Theakston RD, Watson SP, Bon C, Laing GD, Zuzel M. Distinct contributions of glycoprotein VI and $\alpha_2\beta_1$ integrin to the induction of platelet protein tyrosine phosphorylation and aggregation. *Arch Biochem Biophys* 2000; 374: 356–362.
133 Goldschmidt-Clermont PJ, Machesky LM, Baldassare JJ, Pollard TD. The actin-binding protein profilin binds to PIP_2 and inhibits its hydrolysis by phospholipase C. *Science* 1990; 247: 1575–1578.
134 Banno Y, Nakashima T, Kumada T, Ebisawa K, Nonomura Y,

Cambridge Books Online © Cambridge University Press, 2009

Nozawa Y. Effects of gelsolin on human platelet cytosolic phosphoinositide-phospholipase C isozymes. *J Biol Chem* 1992; 267: 6488–6494.
135 Baldassare JJ, Henderson PA, Tarver A, Fisher GJ. Thrombin activation of human platelets dissociates a complex containing gelsolin and actin from phosphatidylinositide-specific phospholipase Cγ1. *Biochem J* 1997; 324: 283–287.
136 Grondin P, Plantavid M, Sultan C, Breton M, Mauco G, Chap H. Interaction of pp60^{c-src}, phospholipase C, inositol-lipid, and diacylglycerol kinases with the cytoskeleton of thrombin-stimulated platelets. *J Biol Chem* 1991; 266: 15705–15709.
137 Guinebault C, Payrastre B, Mauco G, Breton M, Plantavid M, Chap H. Rapid and transient translocation of PLC-γ1 to the cytoskeletons of thrombin-stimulated platelets. Evidence for a role of tyrosine kinases. *Cell Mol Biol* 1994; 40: 687–693.
138 Banno Y, Nakashima S, Ohzawa M, Nozawa Y. Differential translocation of phospholipase C isozymes to integrin-mediated cytoskeletal complexes in thrombin-stimulated human platelets. *J Biol Chem* 1996; 271: 14989–14994.
139 Bleasdale JE, Thakur NR, Gremban RS et al. Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. *J Pharmacol Exp Ther* 1990; 255: 756–768.
140 Heemskerk JWM, Farndale RW, Sage SO. Effects of U73122 and U73343 on human platelet calcium signalling and protein tyrosine phosphorylation. *Biochim Biophys Acta* 1997; 1355: 81–88.
141 Pulcinelli FM, Gresele P, Bonuglia M, Gazzaniga PP. Evidence for separate effects of U73122 on phospholipase C and calcium channels in human platelets. *Biochem Pharmacol* 1998; 56: 1481–1484.
142 Lockhart LK, McNicol A. The phospholipase C inhibitor U73122 inhibits phorbol ester-induced platelet activation. *J Pharmacol Exp Ther* 1999; 289: 721–728.
143 Frohman MA, Sung TC, Morris AJ. Mammalian phospholipase D structure and regulation. *Biochim Biophys Acta* 1999; 1439: 175–186.
144 Exton JH. Signalling through phosphatidylcholine breakdown. *J Biol Chem* 1990; 265: 1–4.
145 Billah MM, Anthes JC. The regulation and cellular functions of phosphatidylcholine hydrolysis. *Biochem J* 1990; 269: 281–291.
146 Petty AC, Scrutton MC. Release of choline metabolites from human platelets: Evidence for activation of phospholipase D and of phosphatidylcholine-specific phospholipase C. *Platelets* 1993; 4: 23–29.
147 Randell E, Mulye H, Mookerjea S, Nagpurkar A. Evidence for phosphatidylcholine hydrolysis by phospholipase C in rat platelets. *Biochim Biophys Acta* 1992; 1124: 273–278.
148 Brown HA, Gutowski S, Moomaw CR, Slaughter C, Sternweis PC. ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. *Cell* 1993; 75: 1137–1144.
149 Pertile P, Liscovitch M, Chalifa V, Cantley LC. Phosphatidylinositol 4,5-bisphosphate synthesis is required for activation of phospholipase D in U937 cells. *J Biol Chem* 1995; 270: 5130–5135.
150 Liscovitch M, Chalifa V, Pertile P, Chen CS, Cantley LC. Novel function of phosphatidylinositol 4,5-bisphosphate as a cofactor for brain membrane phospholipase D. *J Biol Chem* 1994; 269: 21403–21406.
151 Olson SC, Bowman EP, Lambeth JD. Phospholipase D activation in a cell-free system from human neutrophils by phorbol 12-myristate 13-acetate and guanosine 5′-O-(3-thiotriphosphate). Activation is calcium dependent and requires protein factors in both the plasma membrane and cytosol. *J Biol Chem* 1991; 266: 17236–17242.
152 Geny B, Fensome A, Cockcroft S. Rat brain cytosol contains a factor which reconstitutes guanine-nucleotide-binding-protein-regulated phospholipase-D activation in HL60 cells previously permeabilized with streptolysin O. *Eur J Biochem* 1993; 215: 389–396.
153 Cockcroft S, Thomas GM, Fensome A et al. Phospholipase D: a downstream effector of ARF in granulocytes. *Science* 1994; 263: 523–526.
154 Kahn RA, Gilman AG. Purification of a protein cofactor required for ADP-ribosylation of the stimulatory regulatory component of adenylate cyclase by cholera toxin. *J Biol Chem* 1984; 259: 6228–6234.
155 Rothman JE, Orci L. Molecular dissection of the secretory pathway. *Nature* 1992; 355: 409–415.
156 Ktistakis NT, Brown HA, Sternweis PC, Roth MG. Phospholipase D is present on Golgi-enriched membranes and its activation by ADP ribosylation factor is sensitive to brefeldin A. *Proc Natl Acad Sci USA* 1995; 92: 4952–4956.
157 Ktistakis NT, Brown HA, Waters MG, Sternweis PC, Roth MG. Evidence that phospholipase D mediates ADP ribosylation factor-dependent formation of Golgi coated vesicles. *J Cell Biol* 1996; 134: 295–306.
158 Zhang GF, Patton WA, Lee FJ et al. Different ARF domains are required for the activation of cholera toxin and phospholipase D. *J Biol Chem* 1995; 270: 21–24.
159 Hammond SM, Jenco JM, Nakashima S et al. Characterization of two alternately spliced forms of phospholipase D1. Activation of the purified enzymes by phosphatidylinositol 4,5-bisphosphate, ADP-ribosylation factor, and Rho family monomeric GTP-binding proteins and protein kinase C-α. *J Biol Chem* 1997; 272: 3860–3868.
160 Bowman EP, Uhlinger DJ, Lambeth JD. Neutrophil phospholipase D is activated by a membrane-associated Rho family small molecular weight GTP-binding protein. *J Biol Chem* 1993; 268: 21509–21512.
161 Kwak JY, Lopez I, Uhlinger DJ, Ryu SH, Lambeth JD. RhoA and a cytosolic 50-kDa factor reconstitute GTPγS-dependent phospholipase D activity in human neutrophil subcellular fractions. *J Biol Chem* 1995; 270: 27093–27098.
162 Malcolm KC, Elliott CM, Exton JH. Evidence for Rho-mediated agonist stimulation of phospholipase D in rat1 fibroblasts. Effects of *Clostridium botulinum* C3 exoenzyme. *J Biol Chem* 1996; 271: 13135–13139.

Cambridge Books Online © Cambridge University Press, 2009

163 Singer WD, Brown HA, Bokoch GM, Sternweis PC. Resolved phospholipase D activity is modulated by cytosolic factors other than Arf. *J Biol Chem* 1995; 270: 14944–14950.
164 Conricode KM, Brewer KA, Exton JH. Activation of phospholipase D by protein kinase C. Evidence for a phosphorylation-independent mechanism. *J Biol Chem* 1992; 267: 7199–7202.
165 Conricode KM, Smith JL, Burns DJ, Exton JH. Phospholipase D activation in fibroblast membranes by the α and β isoforms of protein kinase C. *FEBS Lett* 1994; 342: 149–153.
166 Lopez I, Burns DJ, Lambeth JD. Regulation of phospholipase D by protein kinase C in human neutrophils. Conventional isoforms of protein kinase C phosphorylate a phospholipase D-related component in the plasma membrane. *J Biol Chem* 1995; 270: 19465–19472.
167 Ohguchi K, Banno Y, Nakashima S, Nozawa Y. Activation of membrane-bound phospholipase D by protein kinase C in HL60 cells: synergistic action of a small GTP-binding protein RhoA. *Biochem Biophys Res Commun* 1995; 211: 306–311.
168 Singer WD, Brown HA, Jiang X, Sternweis PC. Regulation of phospholipase D by protein kinase C is synergistic with ADP-ribosylation factor and independent of protein kinase activity. *J Biol Chem* 1996; 271: 4504–4510.
169 Massenburg D, Han JS, Liyanage M et al. Activation of rat brain phospholipase D by ADP-ribosylation factors 1,5, and 6: separation of ADP-ribosylation factor-dependent and oleate-dependent enzymes. *Proc Natl Acad Sci USA* 1994; 91: 11718–11722.
170 Hammond SM, Altshuller YM, Sung TC et al. Human ADP-ribosylation factor-activated phosphatidylcholine-specific phospholipase D defines a new and highly conserved gene family. *J Biol Chem* 1995; 270: 29640–29643.
171 Sciorra VA, Rudge SA, Prestwich GD, Frohman MA, Engebrecht J, Morris AJ. Identification of a phosphoinositide binding motif that mediates activation of mammalian and yeast phospholipase D isoenzymes. *EMBO J* 1999; 20: 5911–5921.
172 Sung T, Zhang Y, Morris AJ, Frohman MA. Structural analysis of human phospholipase D1. *J Biol Chem* 1999; 274: 3659–3666.
173 Colley WC, Sung T, Roll R et al. Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. *Curr Biol* 1997; 7: 191–201.
174 Sung T, Altshuller YM, Morris AJ, Frohman MA. Molecular analysis of mammalian phospholipase D2. *J Biol Chem* 1999; 274: 494–502.
175 Rubin R. Phosphatidylethanol formation in human platelets: evidence for thrombin-induced activation of phospholipase D. *Biochem Biophys Res Commun* 1988; 156: 1090–1096.
176 Martinson EA, Scheible S, Greinacher A, Presek P. Platelet phospholipase D is activated by protein kinase C via an integrin αIIbβ3-independent mechanism. *Biochem J* 1995; 310: 623–628.
177 Van der Meulen J, Haslam RJ. Phorbol ester treatment of intact rabbit platelets greatly enhances both the basal and guanosine 5′-[γ-thio]triphosphate-stimulated phospholipase D activities of isolated platelet membranes. Physiological activation of phospholipase D may be secondary to activation of phospholipase C. *Biochem J* 1990; 27: 693–700.
178 Huang R, Kucera GL, Rittenhouse SE. Elevated cytosolic Ca^{2+} activates phospholipase D in human platelets. *J Biol Chem* 1991; 266: 1652–1655.
179 Chiang TM. Activation of phospholipase D in human platelets by collagen and thrombin and its relationship to platelet aggregation. *Biochim Biophys Acta* 1994; 1224: 147–155.
180 Martinson EA, Scheible S, Marx-Grunwitz A, Presek P. Secreted ADP plays a central role in thrombin-induced phospholipase D activation in human platelets. *Thromb Haemost* 1998; 80: 976–981.
181 Coorssen JR, Haslam RJ. GTPγS and phorbol ester act synergistically to stimulate both $Ca(^{2+})$-independent secretion and phospholipase D activity in permeabilized human platelets. Inhibition by BAPTA and analogues. *FEBS Lett* 1993; 316: 170–174.
182 Haslam RJ, Coorssen JR. Evidence that activation of phospholipase D can mediate secretion from permeabilized platelets. *Adv Exp Med Biol* 1993; 344: 149–164.
183 Fan XT, Sherwood JL, Haslam RJ. Stimulation of phospholipase D in rabbit platelet membranes by nucleoside triphosphates and by phosphocreatine: roles of membrane-bound GDP, nucleoside diphosphate kinase and creatine kinase. *Biochem J* 1994; 299: 701–709.
184 Nofer JR, Walter M, Kehrel B, Seedorf U, Assmann G. HDL_3 activates phospholipase D in normal but not in glycoprotein IIb/IIIa-deficient platelets. *Biochem Biophys Res Commun* 1995; 207: 148–154.
185 Martinson EA, Scheible S, Presek P. Inhibition of phospholipase D of human platelets by protein tyrosine kinase inhibitors. *Cell Mol Biol* 1994; 40: 627–634.
186 Nakashima S, Suganura A, Matsui A, Nozawa Y. Thrombin induces a biphasic 1,2-diacylglycerol production in human platelets. *Biochem J* 1991; 275: 355–361.

Cambridge Books Online © Cambridge University Press, 2009

17

Platelet signalling: calcium

Juan A. Rosado and Stewart O. Sage

Department of Physiology, University of Cambridge, UK

Introduction

An increase in the cytosolic calcium concentration $[Ca^{2+}]_i$ is a major signal underlying platelet activation[1–4]. Physiological agonists increase the $[Ca^{2+}]_i$ by stimulating both the release of Ca^{2+} from intracellular stores in the endoplasmic reticulum (ER) and the entry of Ca^{2+} across the plasma membrane (PM). After stimulation, the $[Ca^{2+}]_i$ can be reduced by sequestration of Ca^{2+} into the ER and extrusion across the PM. The Ca^{2+}-ATPases responsible also maintain the $[Ca^{2+}]_i$ around 30–50 nM in resting platelets by working against the leakage of Ca^{2+} into the cytosol from the ER and across the PM.

The release of stored Ca^{2+}

Most platelet agonists are able to release stored Ca^{2+} from the ER (the dense tubular system of platelets). The occupation of surface receptors results in the activation of phospholipase C (PLC) and so the formation of the Ca^{2+} releasing messenger, inositol 1,4,5-trisphosphate (IP_3), which acts on receptors on the ER. Many platelet agonists, including ADP, platelet activating factor (PAF), thrombin and thromboxane A_2, have been shown to activate PLC-β_1 via a heterotrimeric GTP-binding protein[3]. PAF and thrombin also appear to activate PLC-γ_1[3], and thrombin and collagen activate PLC-γ_2[5–7].

Platelets are reported to express three isoforms of the IP_3 receptor (IP_3R): IP_3R types I, II and III[8,9]. IP_3RI has been located in the intracellular membranes[8,9], in contrast to IP_3RIII which has been found exclusively at the PM[9]. The IP_3RII has been found associated with both internal membranes and the PM[8,9].

IP_3R function is influenced by a number of variables other than the $[IP_3]$. These include the $[Ca^{2+}]$ at both the cytosolic and lumenal faces of the receptor[10], and cAMP and cGMP via phosphorylation of the IP_3R by protein kinases A and G[11]. Increasing $[Ca^{2+}]$ at the lumenal face of the receptor increases sensitivity to IP_3[12], while at the cytosolic face increased, and then decreased, sensitivity are observed as $[Ca^{2+}]$ increases[13]. Modulation of the sensitivity of the IP_3R to IP_3 may underlie the complex spiking Ca^{2+} signals evoked in platelets by some agonists such as ADP[14]. The inhibitory effects of raising cAMP and cGMP levels on Ca^{2+} signalling contribute the actions of the endothelial products prostacyclin and nitric oxide in limiting platelet activity in vivo[4].

Calcium entry

The release of the finite intracellular Ca^{2+} store is insufficient for full platelet activation, which, in addition, requires Ca^{2+} entry from the external medium[2]. The participation of voltage-operated Ca^{2+} channels in Ca^{2+} influx in platelets is controversial. It is generally accepted that agonists evoke only small depolarizations (5–10 mV) of the platelet membrane potential[15] and eliminating depolarization by replacing external Na^+ with impermeant ions does not affect agonist-evoked Ca^{2+} signalling[16]. However, it has been shown that treatment with L-type Ca^{2+} channel blockers, such as nisoldipine, inhibits agonist-evoked Ca^{2+} influx in platelets[17]. Furthermore, the effect of canatoxin, a toxin protein isolated from *Canavalia ensiformis* seeds that induces Ca^{2+} influx without mobilizing Ca^{2+} from the stores, can be prevented by treatment with voltage-operated Ca^{2+} channel blockers[18]. On the other hand, induction of large membrane depolarizations using high K^+ concentrations does not affect resting $[Ca^{2+}]_i$[16]. Hence Ca^{2+} entry in platelets is mainly activated by receptor occupation by means other than membrane potential change. Receptor-

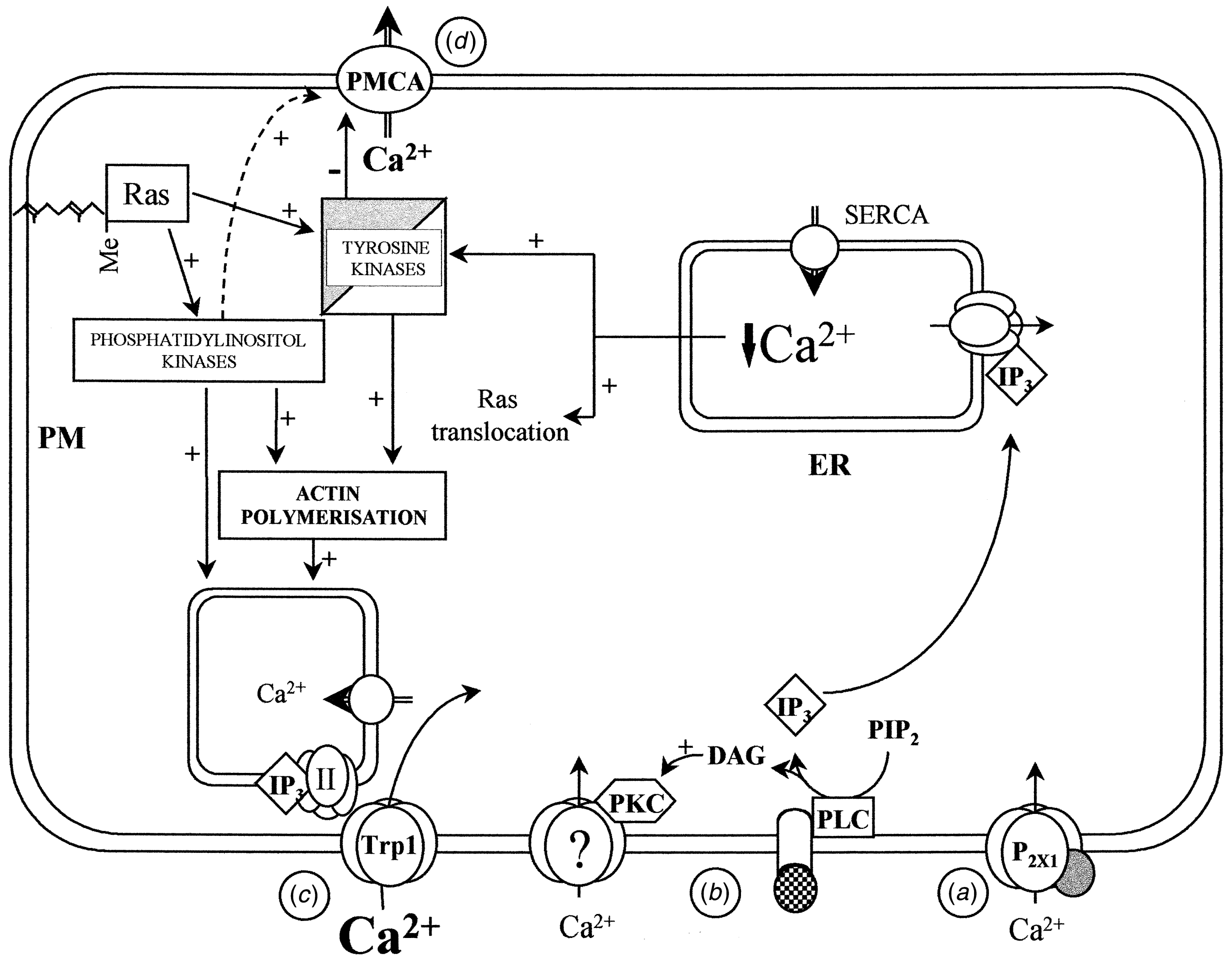


Fig. 17.1. Proposed mechanisms for calcium entry and extrusion in human platelets. (*a*) Adenine nucleotides evoke Ca^{2+} entry via the P_{2X1} purinoceptor. (*b*) Agonist-activated PKC-induced store-independent Ca^{2+} entry via an unidentified channel. (*c*) Store-mediated Ca^{2+} entry. Depletion of the intracellular Ca^{2+} stores induces activation of both unidentified tyrosine kinases and Ras proteins, which, in turn, induce the activation of phosphatidylinositol 3′ and 4′ kinases. Ras, phosphatidylinositol kinases and tyrosine kinases promote actin cytoskeleton rearrangement leading the trafficking of the endoplasmic reticulum (ER) towards the plasma membrane (PM). Reorganized actin filaments support the coupling between elements in the endoplasmic reticulum (IP_3 receptors type II) and Trp1 channels in the plasma membrane through a protein-protein interaction. (*d*) Calcium extrusion via the plasma membrane Ca^{2+} ATPase (PMCA). Activation of Ras proteins stimulates tyrosine phosphorylation of PMCA, which, in turn, inhibits PMCA activity resulting in sustained elevations in $[Ca^{2+}]_i$ required for the activation of Ca^{2+}-dependent events and refilling of the intracellular Ca^{2+} stores. In contrast, activation of phosphatidylinositol kinases increases PMCA activity.

mediated Ca^{2+} entry may take a number of forms[4], several of which appear to be present in platelets. Adenine nucleotides are able to initiate true receptor-operated Ca^{2+} entry, where the Ca^{2+} channel forms part of the P_{2X1} receptor protein[19] (Fig. 17.1(*a*)). Thrombin has been reported to activate a form of second messenger-operated Ca^{2+} entry, which involves diacylglycerol (DAG)[20] (Fig. 17.1(*b*)). However, present evidence indicates that the dominant mechanism employed by platelet agonists is store-mediated Ca^{2+} entry (SMCE), which is activated following depletion of the intracellular Ca^{2+} stores (Fig. 17.1(*c*)).

Ca^{2+} entry via the P_{2X1} purinoceptor

Studies of the early kinetics of agonist-evoked rises in $[Ca^{2+}]_i$, using stopped-flow fluorimetry with platelets loaded with the fluorescent indicator fura-2, revealed that ADP was the only platelet agonist that might act on a true

Cambridge Books Online © Cambridge University Press, 2009

receptor-operated channel[21,22]. In the presence of external Ca^{2+}, ADP was found to evoke a rise in $[Ca^{2+}]_i$ without measurable delay (<20 ms at 37 °C), while release from intracellular stores took some 200 ms at optimal agonist concentrations[22]. Maximally effective concentrations of other agonists, such as thrombin, only evoked Ca^{2+} entry after a latency of around 200 ms[22].

The rapid onset of ADP-evoked Ca^{2+} entry suggested that the ADP receptor might be directly coupled to a Ca^{2+} permeable channel. This was confirmed by the first patch clamp study of stimulated platelets. Cell attached patch clamp recordings revealed little or no activity in resting platelets. ADP was able to evoke single channel currents when added to the pipette filling solution but not when added to the bath[23]. These observations indicated the receptor-operated nature of the Ca^{2+} permeable channel and excluded any role for a diffusible messenger. Ionic substitution experiments indicated that ADP activated a non-selective cation channel, which was demonstrated to be permeable to the Ca^{2+} surrogate Ba^{2+}, and therefore presumably to Ca^{2+} itself[23]. Whole-cell patch clamp recordings of the ADP-evoked current indicated a latency as short as 7 ms[24], in good agreement with estimates of the latency of the ADP-evoked rise in $[Ca^{2+}]_i$[22].

It is now clear that Ca^{2+} entry can be rapidly activated in platelets by several adenine nucleotides including ATP[19] and diadenosine tetraphosphate[25], both of these being agents secreted by activated platelets themselves[26]. Pharmacological studies indicate that the receptor concerned is the ionotropic P_{2X1} receptor, which can be selectively stimulated by $\alpha\beta$-methylene ATP[19]. The presence of mRNA for the P_{2X1} receptor (identical to that first cloned from human bladder[27]) has been demonstrated in platelets using the polymerase chain reaction[28,29] and the expression of the receptor itself has been confirmed by Western blotting[29].

Selective stimulation of the P_{2X1} receptor evokes a relatively small rise in $[Ca^{2+}]_i$, which is also transient because of rapid desensitization[19]. This has led to the physiological significance of the P_{2X1} receptor being questioned. However, it seems the early Ca^{2+} entry via the P_{2X1} receptor has modulatory effects on the subsequent Ca^{2+} signalling evoked via the metabotropic (PLC-activating) P_{2Y1} receptor, which is responsible for ADP-evoked release of Ca^{2+} from intracellular stores[30]. The latency of this IP_3-mediated Ca^{2+} release from intracellular stores is increased when Ca^{2+} entry via the P_{2X1} receptor is prevented[31]. This can be explained by a rise in $[Ca^{2+}]_i$ sensitizing the IP_3 receptor such that release is activated at a lower $[IP_3]$ (see p. 00). Furthermore, the magnitude of ADP-evoked Ca^{2+} signals is reduced if the contribution of the P_{2X1} receptor is eliminated by prior desensitization with $\alpha\beta$-methylene ATP[32]. This effect is particularly pronounced if the levels of the inhibitory cyclic nucleotides, cAMP or cGMP, are elevated, as would be expected in vivo under the influence of the endothelial products, prostacyclin and nitric oxide. This role of the P_{2X1} receptor in enhancing ADP-evoked Ca^{2+} signalling is, like the effect on the kinetics of the response, likely to be due to sensitization of the IP_3 receptor to its ligand and a reduction in the inhibitory effects of IP_3 receptor phosphorylation by protein kinases A and G[11].

Recently, it has been reported that ADP is not an agonist at the platelet P_{2X1} receptor, and that earlier observations can be ascribed to contaminating ATP[33]. This work conflicts, however, with reports that platelets express a splice variant of the P_{2X1} receptor at which ADP is a more potent agonist than other adenine nucleotides[34]. This issue requires further investigation. Even if ADP is not active at the P_{2X1} receptor, this does not diminish its likely physiological significance, since platelets would be expected to encounter several adenine nucleotides together in vivo, notably ADP, ATP and diadenosine tetraphosphate in secretions from activated platelets themselves[26].

Second messenger-evoked Ca^{2+} entry

As in many cells, evidence for second messenger-operated channels (SMOCs) playing a role in platelet Ca^{2+} signalling is rather scarce. The suggestion that inositol phosphates may activate Ca^{2+} permeable channels in the platelet plasma membrane[35] has not gained wide support. However, these reports deserve re-examination in the light of evidence that IP_3 and the IP_3R are involved in the activation of SMCE (p. 265).

Recently, thrombin has been reported to activate Ca^{2+} entry in platelets under conditions which block SMCE[20]. This store-independent (non-capacitative) Ca^{2+} entry was not affected by prior desensitization of the P_{2X1} receptor, indicating that it was not mediated by thrombin-evoked secretion of adenine nucleotides, but it was abolished by inhibitors of protein kinase C (PKC). Since activators of protein kinase C could also evoke Ca^{2+} entry under conditions when SMCE was prevented, it appears that thrombin activates store-independent Ca^{2+} entry via the stimulation of PLC, the formation of DAG and so the activation of PKC. It is possible that other platelet agonists that activate PLC also activate store-independent Ca^{2+} entry in the same manner as thrombin. However, the magnitude of the Ca^{2+} entry activated by thrombin or direct activation of PKC is not large, and since thrombin is the most potent activator of PKC in platelets[36], the contribution of this pathway to the Ca^{2+} signals evoked by other agonists is likely to be small.

Cambridge Books Online © Cambridge University Press, 2009

The presence of a Ca^{2+} entry pathway, independent of Ca^{2+} store depletion, does not necessarily imply the presence of an entirely independent mechanism. Products of human homologues of the *Drosophila* transient receptor potential gene (hTrp) are reported to form Ca^{2+}-permeable channels. One of these, hTrp1, appears to be involved in SMCE in platelets (p. 265). Other Trps have been reported to form channels activated in the presence of DAG lipase inhibitors and by DAG analogues[37]. It may be that hTrp1 represents a point of convergence in store-dependent, and store-independent, PKC-mediated Ca^{2+} entry pathways in platelets.

Store-mediated Ca^{2+} entry

Communication between ER and PM

Although adenine nucleotides have been shown to generate Ca^{2+} entry via ionotropic P_{2X1} purinoceptors, which form non-selective cation channels (pp. 00), and thrombin has been shown to activate Ca^{2+} entry via the formation of diacylglycerol and the activation of protein kinase C (p. 261), the major mechanism generating Ca^{2+} entry in platelets as in other non-excitable cells appears to be that activated following depletion of the intracellular Ca^{2+} stores. The mechanism underlying the activation of this store-mediated Ca^{2+} entry (SMCE) is poorly understood.

A number of hypotheses have been put forward to explain how depletion of the intracellular Ca^{2+} stores might be communicated to the PM to activate Ca^{2+} entry. These can be divided into those that propose a role for a diffusible messenger and those that propose a direct interaction between proteins in the ER and PM (conformational coupling)[3,4,38,39]. Suggestions for diffusible messengers that might be involved in the activation of SMCE have included cyclic GMP[40], a product of cytochrome P450[41], a non-protein Ca^{2+} influx factor (CIF)[42], a tyrosine phosphorylation-dependent step[43–45], small GTP-binding proteins[46–49], the insertion of channels by intracellular trafficking and vesicle fusion[50], and a Ca^{2+}-calmodulin dependent step[51,52]. Conformational coupling models suggest an interaction between the IP_3 receptor (IP_3R) in the membrane of the ER and a Ca^{2+}-permeable channel in the PM[38,53].

Evidence for conformational coupling activating SMCE in platelets

Recently, work in several non-excitable cell types has started to favour conformational coupling as the mechanism responsible for the activation of SMCE[54–56]. These studies have led to the proposal of a secretion-like coupling model, with Ca^{2+} store depletion leading to trafficking of portions of the ER towards the PM to enable the coupling of proteins in the two membranes. Work in platelets indicates an important role for remodelling of the actin cytoskeleton in these events[56], and has provided perhaps the clearest evidence yet for Ca^{2+} store depletion leading to the coupling of an IP_3R in the ER to a Ca^{2+} channel in the PM[57].

Platelet activation involves a morphological transformation of the cells from smooth discs to irregular shapes which have multiple projections. This shape change is mediated by reorganization of the actin cytoskeleton, which is composed of a cytoplasmic actin network and a membrane associated cytoskeleton[58]. If activation of SMCE in platelets occurs following trafficking of the ER towards the PM, to enable coupling between proteins in the two membranes, one might expect this to require reorganization of the actin cytoskeleton. Recent work supports this notion.

SMCE can be activated in platelets by treatment with thapsigargin, an inhibitor of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)[59]. With the Ca^{2+}-ATPase blocked, the resting leak of Ca^{2+} from the ER goes unopposed and results in depletion of the Ca^{2+} store[60]. Depletion of the Ca^{2+} store results in an increase in platelet F-actin content, an effect which is largely independent of any rise in the $[Ca^{2+}]_i$ since it is seen when the Ca^{2+} stores are depleted in platelets heavily loaded with the Ca^{2+} chelator dimethyl BAPTA such that any detectable change in $[Ca^{2+}]_i$ is prevented[61]. Inhibition of actin filament polymerization using the cell-permeant toxins cytochalasin D (which binds to the barbed ends of actin filaments) or latrunculin A (which binds to G actin monomers) has a biphasic effect with time on thapsigargin-evoked SMCE[49,56]. A brief (1 min) preincubation with these agents increased SMCE with only slight inhibition of thapsigargin-evoked actin polymerisation. Longer treatments with the inhibitors (40 min) resulted in complete abolition of thapsigargin-evoked actin polymerization and reduced SMCE by about 50%.

The initial increase in SMCE evoked by cytochalasin D and latrunculin A may be explained by the presence of a dense cortical actin layer in platelets, which is involved in the maintenance of the discoid shape. As the toxins diffuse into the cell, it is likely that they initially affect actin filaments near the plasma membrane. Disruption of cortical actin filaments might be expected to facilitate coupling between the ER and PM, in a manner similar to that whereby actin filament disassembly potentiates secretory exocytosis by enhancing secretory granule docking with the PM[62]. Confocal microscopy of platelets in which F-actin

Cambridge Books Online © Cambridge University Press, 2009

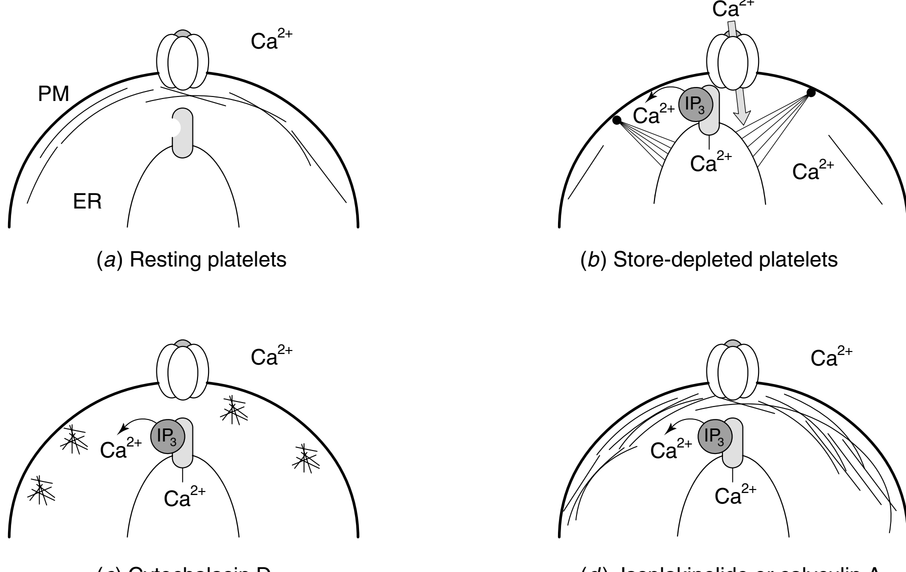


Fig. 17.2. Schematic representation of cytoskeletal organization and its effect on store-mediated Ca^{2+} entry. (*a*) Resting platelets where cortical actin filaments are the main component of the membrane skeleton. (*b*) Store-mediated Ca^{2+} entry in normal platelets mediated via physical coupling between IP_3R type II in the endoplasmic reticulum (ER) and Trp1 channels in the plasma membrane (PM). The coupling is supported by the actin cytoskeleton. (*c*) Cytochalasin D inhibits actin polymerisation and induces reorganisation of the actin filaments into dense foci, preventing the transport of portions of the ER towards the PM and the subsequent coupling. (*d*) Jasplakinolide induces actin polymerization and stabilization of actin filaments into a dense cortical layer displacing cortical ER and blocking store-mediated Ca^{2+} entry. Cytochalasin D and jasplakinolide do not modify IP_3-induced Ca^{2+} release.

has been stained with fluorescein isothiocyanate (FITC)-labelled phalloidin reveals that cytochalasin D results in reorganization of the cortical actin layer into dense foci[63]. This may well enhance ER-PM contact and hence the coupling of proteins therein[63]. These observations in platelets contrast with those reported in NIH 3T3 fibroblasts and in smooth muscle cell lines, where actin filament disruption was without effect on Ca^{2+} entry[54,64]. These differences may reflect the more even distribution of the actin cytoskeleton in fibroblasts and smooth muscle cells. In vascular endothelial cells and astrocytes, cortical actin is involved in maintaining a flat cell morphology[65,66] as it is in platelets[67]. In these cells, too, agents that affect the actin cytoskeleton influence SMCE[68,69].

Other observations also indicate a role for actin filament reorganization in the activation of SMCE in platelets. Agents which stabilize the cortical actin layer inhibit SMCE. Treatment of platelets with the cell-permeant peptide, jasplakinolide, which induces actin polymerization and stabilizes F-actin, increased platelet actin filament content by 100%[56] and resulted in the formation of a dense cortical actin layer[63]. Jasplakinolide treatment resulted in substantial inhibition of SMCE. Similar results were obtained with calyculin A[56], an inhibitor of protein phosphatases 1 and 2, which results in actin polymerization by facilitating the phosphorylation and so activation of the actin-binding proteins, ezrin, radixin and moesin. Fig. 17.2 provides a summary of the cytoskeletal modifications and their effects in the activation of SMCE in human platelets. These results, and those of similar studies in smooth muscle cell lines[54], provide clear evidence against the participation of a diffusible messenger in the activation of SMCE. Such a messenger would be expected to penetrate the actin filament network to activate Ca^{2+} entry, in the same way as IP_3 formed after stimulation by physiological agonists was still able to reach the ER to release stored Ca^{2+} in jasplakinolide-treated cells[54,56].

The actin cytoskeleton appears to play a role in the maintenance as well as in the activation of SMCE in platelets. If platelets are treated with cytochalasin D or latrunculin A after the intracellular Ca^{2+} stores have been depleted using thapsigargin, SMCE is completely inhibited. This inhibition is prevented by jasplakinolide[56]. These results suggest that the actin cytoskeleton may stabilize the coupling between the ER and PM.

Other agents that affect actin filament reorganization also affect SMCE in platelets. Members of the Ras superfamily of small GTP-binding proteins regulate the organ-

Cambridge Books Online © Cambridge University Press, 2009

ization of the actin cytoskeleton in a number of cell types. For example, Rho mediates the assembly of actin filaments and focal adhesion plaques[70,71] and Rac modulates actin filament accumulation by the plasma membrane[72–74]. Farnesylcysteine analogues, which inhibit the prenylation and so membrane association and activation of Ras proteins, reduce both actin polymerization and SMCE in platelets[49]. However, since farnesylcysteine analogues are more effective than agents like cytochalasin at inhibiting SMCE when evoking a similar degree of inhibition of actin polymerization, it appears that small GTP-binding proteins have effects on SMCE which are both dependent on, and independent of, the actin cytoskeleton[49].

Phosphoinositides also play a role in the reorganization of the actin filament network in a number of cell types including platelets[58]. For example, the phosphatidylinositol phosphates PtdIns-3–*P*, PtdIns-3,4–P_2 and PtdIns-3,4,5–P_3 can induce actin filament uncapping and so facilitate actin polymerization[75], and PtdIns-4–*P* and PtdIns-4,5–P_2 regulate cytoskeletal rearrangement by associating with actin binding proteins[76]. Inhibiting phosphoinositide 3-kinase (PI3-kinase) and phosphoinositide 4-kinase (PI4-kinase) using LY294002 or wortmannin, so reducing phosphatidylinositol phosphate levels, results in the inhibition of both actin polymerization and SMCE in platelets[77]. PI3- and PI4-kinases have been shown to be regulated upstream by small GTP-binding proteins such as Rac and Rho[71,78]. Hence Ras proteins and phosphoinositide kinases may be sequentially involved in the activation of SMCE[63].

Reports that inhibitors of tyrosine kinases can inhibit SMCE in platelets[43,44,79] may be explained by the roles of tyrosine kinases in the reorganization of the actin cytoskeleton[61]. The tyrosine kinase inhibitor methyl 2,5-dihydroxycinnamate can completely inhibit agonist or Ca^{2+} store depletion-evoked actin polymerization in platelets and results in a substantial reduction in SMCE[61]. The effect of the tyrosine kinase inhibitor was not additive with the inhibitory effect of the cytoskeletal disrupter cytochalasin D, indicating that the action of the tyrosine kinase inhibitor might be mediated entirely via effects on the reorganization of the actin cytoskeleton.

Thus there is a body of evidence supporting a role for reorganization of the actin cytoskeleton in the activation of SMCE in platelets. These data are compatible with a secretion-like coupling model in which Ca^{2+} store depletion results in the trafficking of a portion of the ER towards the PM. As yet, however, there is no ultrastructural evidence for the trafficking event itself.

The proteins responsible for conformational coupling in platelets

Recent work has confirmed the requirement for IP_3 and functional IP_3Rs in both the activation and maintenance of SMCE in platelets[57]. Blocking IP_3 recycling by treating platelets with Li^+ for 2 h completely eliminated thrombin-evoked release of Ca^{2+} from intracellular stores, indicating that IP_3 production had ceased. The application of thapsigargin together with the Ca^{2+} ionophore, ionomycin, was still able to elevate $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, confirming that the Ca^{2+} stores were intact. Li^+ treatment completely blocked SMCE in thapsigargin-treated (Ca^{2+} store depleted) platelets. If the Ca^{2+} stores were depleted using thapsigargin prior to treatment with Li^+, the subsequent addition of extracellular Ca^{2+} did not result in SMCE, indicating that IP_3 is required for both the activation and maintenance of SMCE. The effects of Li^+ could be reversed by co-incubation with *myo*-inositol, indicating that Li^+ was acting by interfering with IP_3 production.

The role of IP_3Rs in SMCE was assessed using xestospongin C, a cell permeant inhibitor of IP_3R function isolated from a marine sponge. Xestospongin C completely blocked thrombin-evoked release of Ca^{2+} from intracellular stores[57]. The same treatment did not affect the ability of thapsigargin to deplete the Ca^{2+} stores, but abolished SMCE. If xestospongin C was added after the Ca^{2+} stores had been depleted using thapsigargin, subsequent Ca^{2+} entry on the addition of external Ca^{2+} was also abolished. Thus functional IP_3Rs are required for both the activation and maintenance of SMCE in platelets.

The conformational coupling hypothesis for the activation of SMCE has proposed from the outset that the coupling involves IP_3Rs in the ER[53]. The nature of the protein or proteins involved in the plasma membrane has always been less certain. A major problem has been the clear identification of the channels which mediate SMCE. For a number of years now attention has focused on mammalian homologues of the *Drosophila* transient receptor potential (Trp) channel proteins. The presence of messenger RNA for human Trp1 (hTrp1) and its splice variant, hTrp1A, has been demonstrated in human platelets (D. Molin, E. den Dekker, G. Breikers, R. van Oerle, J.W. Akkerman & J.W.M. Heemskerk, personal communication), as has the expression of hTrp1 in these cells[57]. These observations led us to investigate the possible coupling of hTrp1 to IP_3Rs in platelets in a series of co-immunoprecipitation experiments.

Platelets are reported to express three isoforms of the IP_3R: IP_3R types I, II and III[8,9]. Coupling between each of these IP_3Rs and hTrp1 was tested for by looking for co-immunoprecipitation from lysates obtained from control

Cambridge Books Online © Cambridge University Press, 2009

(resting) platelets and platelets in which the intracellular Ca^{2+} stores had been depleted by treatment with thapsigargin[57]. After immunoprecipitation with anti-hTrp1, SDS–PAGE and Western blotting revealed the presence of IP_3RII in immunoprecipitates from store-depleted but not control platelets. IP_3RI and IP_3RII were undetectable in samples from both control and depleted cells. Converse experiments were also conducted in which platelet lysates were immunoprecipitated with anti-IP_3RI, anti-IP_3RII or anti-IP_3RIII antibodies and then Western blots were probed for the presence of hTrp1. Following immunoprecipitation with anti-IP_3RI or anti-IP_3RIII, hTrp1 was undetectable in samples from control and Ca^{2+} store-depleted platelets; however, hTrp1 was detected in immunoprecipitates obtained with anti-hTrp1 from store-depleted but not control cells[57]. These results indicate that only homotetramers of IP_3RII couple with hTrp1 upon Ca^{2+} store depletion in platelets, and that IP_3RI and IP_3RIII are not involved in the conformational coupling mechanism. Although it might be argued that the failure to detect coupling of hTrp1 with two of the IP_3R isoforms is due to low levels of expression and/or lower antibody sensitivity, the results are in good agreement with reports that only IP_3RII is located in both the plasma membrane and the intracellular membranes in platelets[8,9], with IP_3RI being restricted to the intracellular membranes[8,9] and IP_3RIII being located only in the plasma membrane[9].

Co-immunoprecipitation experiments not only provide strong support for conformational coupling underlying the activation of SMCE in platelets, but also indicate that hTrp1 is involved in forming the channels which mediate Ca^{2+} entry. Human Trp1 has been reported to form non-selective cation channels in other cells[80]. Whether hTrp1 alone conducts SMCE in platelets, or whether it forms heteromeric structures with hTrp1A or other proteins, remains to be determined. Coupling between various hTrp and IP_3R isoforms has been reported in other cell types[81–83]. In these studies in various transfected cell lines, the coupling appeared to be constitutive and independent of intracellular Ca^{2+} store depletion. Thus in these transfected cells, there appeared to be relatively stable interactions between IP_3Rs and hTrps, with channel activity presumably depending on changes in the concentration of IP_3 and/or the Ca^{2+} content of the store. These results contrast with the observations in platelets, where there is clear evidence for coupling between endogenously expressed IP_3RII and hTrp1, and for this coupling occurring *de novo* when the intracellular Ca^{2+} stores are depleted[57].

Recent work thus provides strong evidence for conformational coupling underlying the activation of SMCE in human platelets. Ca^{2+} store depletion leads to reorganization of the actin cytoskeleton, an event which appears to involve small GTP-binding proteins and phosphoinositides. These cytoskeletal changes appear necessary to permit, and may mediate, the events required to allow the coupling of elements in the ER and PM to occur. In accordance with the secretion-like coupling model for SMCE, these events are envisaged as a trafficking of portions of the ER towards the PM. Coupling itself appears to involve just one isoform of IP_3R, IP_3RII, and hTrp1. The tentative scheme we propose is illustrated in Fig. 17.1(*c*). Whether other proteins are also involved in the coupling process, and the precise relationship between the initial Ca^{2+} store depletion and the events outlined, remain to be determined.

Regulatory effect of integrin $\alpha_{IIb}\beta_3$ and adrenaline on Ca^{2+} influx

Integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa) is a member of the integrin family of receptors that mediate cellular adhesion of different cell types to several ligands, such as fibrinogen, fibronectin and von Willebrand factor[84,85]. Occupancy of specific sites of the integrin $\alpha_{IIb}\beta_3$ by a ligand affects its structure, function and distribution[85]. It has been shown that peptides containing the Arg–Gly–Asp sequence, known as the RGD sequence, present in the A-α chains of human fibrinogen, bind to potential recognition sites on the integrin $\alpha_{IIb}\beta_3$ for the binding of soluble fibrinogen on activated human platelets, thus inhibiting binding of fibrinogen with the receptor[86,87]. Platelet agonists activate integrin $\alpha_{IIb}\beta_3$ (inside-out signalling) to allow the binding of soluble fibrinogen followed by outside-in signals involving phosphorylation events, cytoskeletal rearrangements and molecular translocation[88]. It has been reported that integrin $\alpha_{IIb}\beta_3$ contains high affinity Ca^{2+} binding sites[89] and might be involved in the regulation of $[Ca^{2+}]_i$ in platelets[84,90–92]; however, the mechanisms responsible have not been clearly addressed in these early reports, nor has whether occupation of the integrin influences Ca^{2+} store depletion or Ca^{2+} influx. Recent studies addressing this issue indicate that the binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$ inhibits the activation of SMCE in platelets by a mechanism which may involve modulation of the reorganization of the actin cytoskeleton: an action that may be important in intrinsic negative feedback to prevent the further activation of platelets subjected to low level stimuli in vivo[93]. In the absence of agonist stimulation, PMA4-induced fibrinogen binding to the integrin $\alpha_{IIb}\beta_3$ does not induce Ca^{2+} influx or mobilization, suggesting that, in the absence of agonist-induced signalling, $\alpha_{IIb}\beta_3$ occupied by fibrinogen generates only a limited outside-in signal[94].

Cambridge Books Online © Cambridge University Press, 2009

It has been suggested that the integrin $\alpha_{IIb}\beta_3$ mediates the synergistic $[Ca^{2+}]_i$ rise that follows addition of both thrombin and adrenaline in human platelets[95]. The role of adrenaline as a $[Ca^{2+}]_i$ elevating agonist in platelets is controversial. Adrenaline has been shown by some authors to elevate $[Ca^{2+}]_i$ in platelets through a mechanism dependent on the presence of extracellular Ca^{2+}[95,96]. Ca^{2+} entry stimulated by adrenaline has been shown to be associated with fibrinogen binding to the integrin $\alpha_{IIb}\beta_3$[97]; however, treatment of platelets with antibodies that prevent fibrinogen binding does not inhibit adrenaline-induced Ca^{2+} influx[95]. Others have reported adenaline alone to be without effect on $[Ca^{2+}]_i$ in platelets[98]. In addition, it has been reported that adrenaline potentiates Ca^{2+} elevation stimulated by platelet agonists, such as ADP[99], thrombin[95,98] or collagen[100], an event that might be mediated by the activation of the $\alpha_{IIb}\beta_3$ integrin and fibrinogen binding[95,96] or by decreases in cAMP[98].

Removal of Ca^{2+} from the cytosol

Ca^{2+} may be removed from the cytosol by uptake into the ER, uptake into mitochondria and by extrusion across the plasma membrane. However, the application of mitochondrial inhibitors to platelets has little or no effect on resting $[Ca^{2+}]_i$[101] or on the return of $[Ca^{2+}]_i$ towards resting levels after an elevation[102]. These observations indicate that the mitochondria do not play a significant role in Ca^{2+} homeostasis in platelets.

The uptake of Ca^{2+} into the ER is required after store discharge in order to allow further signalling if platelets are not irreversibly activated and to allow the periodic discharge of Ca^{2+} which underlies agonist-evoked Ca^{2+} spiking[14,103]. Platelets express several isoforms of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), with SERCA 2b[104], SERCA 3a[105,106], SERCA 3b[107] and SERCA 3c[108] all being reported. SERCA 3b appears to be associated with the IP_3-releasible Ca^{2+} store in platelets[109].

As well as being responsive to changes in $[Ca^{2+}]_i$, SERCA activity may be modulated by other factors which participate in the overall regulation of platelet Ca^{2+} signalling. For example, the inhibitory effects of elevated cAMP may, in part, be mediated by stimulated uptake of Ca^{2+} into intracellular stores[110,111]. The mechanism may involve the small GTP-binding protein, Rap 1b[112]. It is suggested that Rap 1b associates with SERCA 3b when Rap 1b is activated by a rise in $[Ca^{2+}]_i$, so inhibiting SERCA 3b activity and potentiating agonist-evoked Ca^{2+} signals. When Rap 1b is phosphorylated following a rise in cAMP, it dissociates from SERCA 3b, increasing its activity and so Ca^{2+} uptake into the intracellular stores[112]. Nitric oxide may also exert its inhibitory effect on platelet function in part by promoting Ca^{2+} uptake into the intracellular stores. This action has been reported as independent of[113], or only partially dependent on[114], increases in cGMP.

Removal of Ca^{2+} from the cell across the plasma membrane can be effected by Na^+/Ca^{2+} exchange or a plasma membrane Ca^{2+}-ATPase (PMCA). Na^+/Ca^{2+} exchange appears to be present in human platelets and the exchanger appears to be of the Na^+–Ca^{2+}–K^+ exchange type[115,116] found in retinal rods. However, the removal of extracellular Na^+ has little if any effect on resting $[Ca^{2+}]_i$[117] or the restoration of $[Ca^{2+}]_i$ after it has been elevated[102,118]. These observations indicate that Na^+/Ca^{2+} exchange plays no major role in normal platelet Ca^{2+} homeostasis and that the PMCA is the major extrusion mechanism across the plasma membrane[102].

Platelets are reported to express two isoforms of PMCA: PMCA 1b and PMCA 4b[108]. As with SERCAs, PMCAs appear to be subject to regulation by a number of signalling systems other than $[Ca^{2+}]_i$. An increase in platelet cAMP levels promotes Ca^{2+} extrusion across the plasma membrane[111] by increasing PMCA activity through phosphorylation of the enzyme[119]. This action is likely to contribute to the inhibitory effect of elevated cAMP on platelet activity. Conversely, inhibition of adenylyl cyclase by platelet agonists such as ADP would be expected to reduce PMCA activity, an action which may explain the reduction in the ADP-evoked Ca^{2+} signal when this mechanism is selectively blocked[32].

PMCA activity in platelets is inhibited by the phosphorylation of the enzyme on tyrosine residues[119]. The increase in protein tyrosine phosphorylation seen upon platelet activation may thus potentiate agonist-evoked Ca^{2+} signals and the activation process. Loss of this action may contribute to the reduction in agonist-evoked elevations in $[Ca^{2+}]_i$ observed in platelets treated with tyrosine kinase inhibitors[43,44]. Tyrosine phosphorylation and so inhibition of platelet PMCA activity appear to lie downstream of the activation of small GTP-binding proteins of the Ras family. Inhibition of Ras activation using farnesylcysteine analogues (p. 264) inhibited the increase in tyrosine phosphorylation of the PMCA seen when the intracellular Ca^{2+} stores in platelets were depleted and accelerated the decline in $[Ca^{2+}]_i$ towards basal levels[102] (Fig. 17.1(*d*)).

Platelet PMCA activity is also modulated by products of PI3-kinase and PI4-kinase. Inhibition of these kinases results in a reduction in the rate of decline in $[Ca^{2+}]_i$ in platelets which have been treated with thapsigargin and ionomycin[102] (Fig. 17.1(*d*)). These observations are consistent with reports that acidic phospholipids, such as

Cambridge Books Online © Cambridge University Press, 2009

PtdIns-4,5-P_2, activate the PMCA and are responsible for PMCA activity in resting cells[120,121].

Acknowledgements

J. A. R. is supported by a Grant of Junta de Extremadura-Consejería de Educación y Juventud and Fondo Social Europeo, Spain. Supported by The Wellcome Trust (051560).

References

1 Siess W. Molecular mechanisms of platelet activation. *Physiol Rev* 1989; 69: 59–178.
2 Rink TJ, Sage SO. Calcium signalling in human platelets. *Annu Rev Physiol* 1990; 52: 429–447.
3 Heemskerk JWM, Sage SO. Calcium signalling in platelets and other cells. *Platelets* 1994; 5: 295–316.
4 Sage SO. The Wellcome Prize Lecture. Calcium entry mechanisms in human platelets. *Exp Physiol* 1997; 82: 807–823.
5 Daniel JL, Dangelmaier C, Smith JB. Evidence for a role for tyrosine phosphorylation of phospholipase C-γ-2 in collagen-induced platelet cytosolic calcium mobilisation. *Biochem J* 1994; 302: 617–622.
6 Blake RA, Schieven GL, Watson SP. Collagen stimulates tyrosine phosphorylation of phospholipase C-γ-2 but not phospholipase C-γ-1 in human platelets. *FEBS Lett* 1994; 353: 212–216.
7 Gibbins J, Asselin J, Farndale R, Barnes M, Law CL, Watson SP. Tyrosine phosphorylation of the Fc receptor gamma-chain in collagen-stimulated platelets. *J Biol Chem* 1996; 271: 18095–18099.
8 Quinton TM, Dean WL. Multiple inositol 1,4,5-trisphosphate receptor isoforms are present in platelets. *Biochem Biophys Res Commun* 1996; 224: 740–746.
9 El-Daher SS, Patel Y, Siddiqua A et al. Distinct localization and function of (1,4,5)IP_3 receptor subtypes and the (1,3,4,5) IP_4 receptor GAP1(IP_4BP) in highly purified human platelet membranes. *Blood* 2000; 95: 3412–3422.
10 Berridge MJ. Annual Review Prize Lecture. Elementary and global aspects of calcium signalling. *J Physiol (Lond)* 1997; 499: 291–306.
11 Cavallini L, Coassin M, Borean A, Alexandre A. Prostacyclin and sodium nitroprusside inhibit the activity of the platelet inositol 1,4,5-trisphosphate receptor and promote its phosphorylation. *J Biol Chem* 1996; 271: 5545–5551.
12 Missiaen L, Taylor CW, Berridge MJ. Spontaneous calcium release from inositol trisphosphate-sensitive calcium stores. *Nature* 1991; 352: 241–244.
13 Bezprozvanny I, Watras J, Ehrlich, BE. Bell-shaped calcium-response curves of Ins(1,4,5)P_3-gated and calcium-gated channels from endoplasmic reticulum of cerebellum. *Nature* 1991; 351: 751–754.
14 Heemskerk JWM, Hoyland J, Mason WT, Sage SO. Oscillations in cytosolic calcium concentration in single, fibrinogen bound, fura-2-loaded human platelets. *Biochem J* 1992; 283: 379–383.
15 Pipili E. Platelet membrane potential: simultaneous measurement of diSC3(5) fluorescence and optical density. *Thromb Haemost* 1985; 54: 645–649.
16 Sage SO, Rink TJ. Effects of ionic substitution on $[Ca^{2+}]_i$-rises evoked by thrombin and PAF in human platelets. *Eur J Pharmacol* 1986; 128: 99–107.
17 Pales J, Palacios-Araus L, Lopez A, Gual A. Effects of dihydropyridines and inorganic calcium blockers on aggregation and on intracellular free calcium in platelets. *Biochim Biophys Acta* 1991; 1064: 169–174.
18 Ghazaleh FA, Francischetti I MB, Gombarovits MEC, Carlini CR. Stimulation of calcium influx and platelet activation by canatoxin: methoxyverapamil inhibition and down regulation by cGMP. *Arch Biochem Biophys* 1997; 339: 362–367.
19 MacKenzie AB, Mahaut-Smith MP, Sage SO. Activation of receptor-operated cation channels via P_{2X1} not P_{2T} purinoceptors in human platelets. *J Biol Chem* 1996; 271: 2879–2881.
20 Rosado JA, Sage SO. Protein kinase C activates non-capacitative calcium entry in human platelets. *J Physiol (Lond)* 2000; 529: 159–169.
21 Sage SO, Rink TJ. Kinetic differences between thrombin-induced and ADP-induced calcium influx and release from internal stores in fura-2-loaded human platelets. *Biochem Biophys Res Commun* 1996; 136: 1124–1129.
22 Sage SO, Rink TJ. The kinetics of changes in intracellular calcium concentration in fura-2-loaded human platelets. *J Biol Chem* 1987; 262: 16364–16369.
23 Mahaut-Smith MP, Sage SO, Rink TJ. Single channels in intact human platelets evoked by ADP. *J Biol Chem* 1990; 265: 10479–10483.
24 Mahaut-Smith MP, Sage SO, Rink TJ. Rapid ADP-evoked currents in human platelets recorded with the nystatin permeabilised patch technique. *J Biol Chem* 1992; 267: 3060–3065.
25 Sage SO, MacKenzie AB, Jenner S, Mahaut-Smith MP. Purinoceptor-evoked calcium signalling in human platelets. *Prostaglandins, Leukotrienes and Essential Fatty Acids* 1997; 57: 435–438.
26 Flodgaard H, Klenow H. Abundant amounts of diadenosine 5′,5″-P1,P4-tetraphosphate are present and releasable, but metabolically inactive, in human platelets. *Biochem J* 1982; 208: 737–742.
27 Surprenant A, Buell G, North RA. P2X receptors bring new structure to ligand-gated ion channels. *Trends Neurosci* 1995; 18: 224–229.
28 Vial C, Hechler B, Leon C, Cazenave JP, Gachet C. Presence of P_{2X1} purinoceptors in human platelets and megakaryoblastic cell lines. *Thromb Haemost* 1997; 78: 1500–1504.

Cambridge Books Online © Cambridge University Press, 2009

29 Scase TJ, Heath MF, Allen JM, Sage SO, Evans RJ. Identification of a P2X1 purinoceptor expressed on human platelets. *Biochem Biophys Res Commun* 1998; 242: 525–528.

30 Jin J, Daniel JL, Kunapuli SP. Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J Biol Chem* 1998; 273: 2030–2034.

31 Sage SO, Reast R, Rink TJ. ADP evokes biphasic Ca^{2+} influx in human platelets: evidence for a store-regulated route of Ca^{2+} entry. *Biochem J* 1990; 265: 675–680.

32 Sage SO, Yamoah EH, Heemskerk JWM. The roles of P_{2X1} and P_{2TAC} receptors in ADP-evoked calcium signalling in human platelets. *Cell Calcium* 2000; 28: 119–126.

33 Mahaut-Smith MP, Ennion SJ, Rolf MG, Evans RJ. ADP is not an agonist at P2X(1) receptors: evidence for separate receptors stimulated by ATP and ADP on human platelets. *Br J Pharmacol* 2000; 131: 108–114.

34 Greco NJ, Tonon, G, Chen WD, Luo XY, Dalal R, Jamieson GA. Identification of a truncated ADP-activated P-2X1 receptor in platelets and megakaryocytic cell lines. *Thromb Haemost* Suppl S 1999; 421.

35 Rengasamy A, Feinberg H. Inositol 1,4,5-trisphosphate-induced calcium release from platelet plasma membrane vesicles. *Biochem Biophys Res Commun* 1988; 150: 1021–1026.

36 Pollock WK. PhD thesis, University of Glasgow, 1984.

37 Ma HT, Patterson RL, Van Rossum DB, Birnbaumer L, Mikoshiba K, Gill DL. Requirement of the inositol trisphosphate receptor for activation of store-operated Ca^{2+} channels. *Science* 2000; 287: 1647–1651.

38 Berridge MJ. Capacitative calcium entry. *Biochem J* 1995; 312: 1–11.

39 Parekh AB, Penner R. Store depletion and calcium influx. *Physiol Rev* 1997; 77: 901–930.

40 Pandol SJ, Schoeffield-Payne MS. Cyclic AMP mediates the agonist-stimulated increase in plasma membrane calcium entry in the pancreatic acinar cell. *J Biol Chem* 1990; 265: 12846–12853.

41 Álvarez J, Montero M, García-Sancho J. Cytochrome P450 may regulate plasma membrane Ca^{2+} permeability according to the filling state of the intracellular Ca^{2+} stores. *FASEB J* 1992; 6: 786–792.

42 Radriamampita C, Tsien RY. Emptying of intracellular Ca^{2+} stores release a novel small messenger that stimulates Ca^{2+} influx. *Nature* 1993; 364: 809–814.

43 Sargeant P, Farndale RW, Sage SO. The tyrosine kinase inhibitors methyl 2,5-dihydroxycinnamate and genistein reduce thrombin-evoked tyrosine phosphorylation and Ca^{2+} entry in human platelets. *FEBS Lett* 1993; 315: 242–246.

44 Sargeant P, Farndale RW, Sage SO. ADP- and thapsigargin-evoked Ca^{2+} entry and protein tyrosine phosphorylation is inhibited by the tyrosine kinase inhibitors genistein and methyl 2,5-dihydroxycinnamate in fura-2-loaded human platelets. *J Biol Chem* 1993; 268: 18151–18156.

45 Jenner S, Farndale RW, Sage SO. Effects of Ca^{2+} store depletion and refilling with Ca^{2+}, Sr^{2+} or Ba^{2+} on tyrosine phosphorylation and Mn^{2+} entry in human platelets. *Biochem J* 1994; 303: 337–339.

46 Fasolato C, Hoth M, Penner R. A GTP-dependent step in the activation mechanism of capacitative calcium influx. *J Biol Chem* 1993; 268: 5545–5551.

47 Bird GStJ, Putney JW. Inhibition of thapsigargin-induced calcium entry by microinjected guanine nucleotide analogues. Evidence for the involvement of a small G-protein in capacitative calcium entry. *J Biol Chem* 1993; 268: 21486–21488.

48 Xu X, Gilbert BA, Rando RR, Chen L, Tashjian AH. Inhibition of capacitative Ca^{2+} entry into cells by farnesylcysteine analogs. *Mol Pharmacol* 1996; 50: 1495–1501.

49 Rosado JA, Sage SO. Farnesylcysteine analogues inhibit store-regulated calcium entry in human platelets. Evidence for involvement of small GTP-binding proteins and actin cytoskeleton. *Biochem J* 2000; 347: 183–192.

50 Somasundaram B, Norman JC, Mahaut-Smith MP. Primaquine, an inhibitor of vesicular transport, blocks the calcium-release-activated current in rat megakaryocytes. *Biochem J* 1995; 309: 725–729.

51 Gailly P, Hermans E, Gillis JM. Role of $[Ca^{2+}]_i$ in Ca^{2+} stores-depletion-Ca^{2+} entry coupling in fibroblasts expressing the rat neurotensin receptor. *J Physiol (Lond)* 1996; 491: 635–646.

52 Vaca L. Calmodulin inhibits calcium influx current in vascular endothelium. *FEBS Lett* 1996; 390: 289–293.

53 Irvine RF. 'Quantal' Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates: a possible mechanism. *FEBS Lett* 1990; 263: 5–9.

54 Patterson RL, Van Rossum DB, Gill DL. Store-operated Ca^{2+} entry: evidence for a secretion-like coupling model. *Cell* 1999; 98: 487–499.

55 Yao Y, Ferrer-Montiel AV, Montal M, Tsien, RY. Activation of store-operated Ca^{2+} current in *Xenopus* oocytes requires SNAP-25 but not a diffusible messenger. *Cell* 1999; 98: 475–485.

56 Rosado JA, Jenner S, Sage SO. A role for the actin cytoskeleton in the initiation and maintenance of store-mediated calcium entry in human platelets. Evidence for conformational coupling. *J Biol Chem* 2000; 275: 7527–7533.

57 Rosado JA, Sage SO. IP_3 receptors couple with hTrp1 channels when Ca^{2+} stores are depleted in human platelets. *Biochem J* 2000; 350: 631–635.

58 Ma AD, Abrams CS. Pleckstrin homology domains and phospholipid-induced cytoskeletal reorganisation. *Thromb Haemost* 1999; 82: 399–406.

59 Thastrup O, Dawson AP, Scharff O et al. Thapsigargin, a novel molecular probe for studying intracellular calcium release and storage. *Agents Actions* 1989; 43: 187–193.

60 Sargeant P, Clarkson WD, Sage SO, Heemskerk JWM. Calcium influx evoked by store depletion in human platelets is more susceptible to cytochrome *P*-450 inhibitors than receptor-mediated calcium entry. *Cell Calcium* 1992; 13: 565–576.

61 Rosado JA, Graves D, Sage SO. Tyrosine kinases activate store-mediated Ca^{2+} entry in human platelets through the reorganisation of the actin cytoskeleton. A role for p60src in the

Cambridge Books Online © Cambridge University Press, 2009

activation of store-mediated calcium entry. *Biochem J* 2000; 351: 429–437.

62 Muallem S, Kwiatkowska K, Xu X, Yin HL. Actin filament disassembly is a sufficient final trigger for exocytosis in nonexcitable cells. *J Cell Biol* 1995; 128: 589–598.

63 Rosado JA, Sage SO. The actin cytoskeleton and store-mediated calcium entry. *J Physiol (Lond)* 2000; 526: 221–229.

64 Pedrosa-Ribeiro CM, Reece J, Putney JW. Role of the cytoskeleton in calcium signalling in NIH 3T3 cells. An intact cytoskeleton is required for agonist-induced $[Ca^{2+}]_i$ signalling, but not for capacitative calcium entry. *J Biol Chem* 1997; 272: 26555–26561.

65 Simonescu N, Simonescu M. Ultrastructure of the microvascular wall: functional correlations. In Weiss L. (ed.) *The cardiovascular system. Histology, cell and tissue biology*, 5th edn, New York: Elsevier Science Publishing Company, Inc; 1983: 371–433.

66 Jones EG, Cowan WM. The nervous tissue. In: Weiss L. *The cardiovascular system. Histology, cell and tissue biology*, 5th edn, New York: Elsevier Science Publishing Company, Inc; 1983: 282–370.

67 Hartwig JH. Mechanisms of actin rearrangements mediating platelet activation. *J Cell Biol* 1992; 118: 1421–1442.

68 Holda JR, Blatter LA. Capacitative calcium entry is inhibited in vascular endothelial cells by disruption of cytoskeletal microfilaments. *FEBS Lett* 1997; 403: 191–196.

69 Grimaldi M, Favit A, Alkon DL. cAMP-induced cytoskeleton rearrangement increases calcium transient through the enhancement of capacitative calcium entry. *J Biol Chem* 1999; 274: 33557–33564.

70 Symonds M. Rho family GTPases: the cytoskeleton and beyond. *Trend Biochem Sci* 1996; 21: 178–181.

71 Fox JEB. On the role of calpain and Rho proteins in regulating integrin-induced signalling. *Thromb Haemost* 1999; 82: 385–391.

72 Ridley AJ, Hall A. Signal transduction pathways regulating Rho-mediated stress fibre formation: requirement for a tyrosine kinase. *EMBO J* 1994; 13: 2600–2610.

73 Nobes CD, Hall A. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 1995; 81: 53–62.

74 Olson MF, Ashworth A, Hall A. An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. *Science* 1995; 269: 1270–1272.

75 Hartwig J H, Kung S, Kovacsovics T et al. D3 phosphoinositides and outside-in integrin signalling by glycoprotein IIb–IIIa mediate platelet actin assembly and filopodial extension induced by phorbol 12-myristate 13-acetate. *J Biol Chem* 1996; 271: 32986–32993.

76 Lassing I, Lindberg U. Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. *Nature* 1985; 314: 472–474.

77 Rosado JA, Sage SO. Phosphoinositides are required for store-regulated calcium entry in human platelets. *J Biol Chem* 2000; 275: 9110–9113.

78 Gasman S, Chasserot-Golaz S, Hubert P, Aunis D, Bader MF. Identification of a potential effector pathway for the trimeric Go protein associated with secretory granules. Go stimulates a granule-bound phosphatidylinositol 4-kinase by activating RhoA in chromaffin cells. *J Biol Chem* 1998; 273: 16913–16920.

79 Vostal JG, Jackson WL, Shulman NR. Cytosolic and stored calcium antagonistically control tyrosine phosphorylation of specific platelet proteins. *J Biol Chem* 1991; 266: 16911–16916.

80 Sinkins WG, Estacion M, Schilling WP. Functional expression of TrpC1: a human homologue of the *Drosophila* Trp channel. *Biochem J* 1998; 331: 331–339.

81 Kiselyov K, Xu X, Mozhayeva G et al. Functional interaction between $InsP_3$ receptors and store-operated Htrp3 channels. *Nature* 1998; 396: 478–482.

82 Boulay G, Brown DM, Qin N et al. Modulation of Ca^{2+} entry by polypeptides of the inositol 1,4, 5-trisphosphate receptor (IP_3R) that bind transient receptor potential (TRP): evidence for roles of TRP and IP_3R in store depletion-activated Ca^{2+} entry. *Proc Natl Acad Sci USA* 1999; 96: 14955–14960.

83 Lockwich TP, Liu X, Singh BB, Jadlowiec J, Weiland S, Ambudkar IS. Assembly of Trp1 in a signalling complex associated with caveolin-scaffolding lipid raft domains. *J Biol Chem* 2000; 275: 11934–11942.

84 Sinigaglia F, Bisio A, Torti M, Balduini CL, Bertolino G, Balduini C. Effect of GPIIb–IIIa complex ligands on calcium ion movement and cytoskeleton organization in activated platelets. *Biochem Biophys Res Commun* 1988; 154: 258–264.

85 Padoin E, Alexandre A, Cavallini L, Polverino de Laureto P, Rao GH, Doni MG. Human platelet activation is inhibited by the occupancy of glycoprotein IIb/IIIa receptor. *Arch Biochem Biophys* 1996; 333: 407–413.

86 Bugaud F, Nadal-Wollbold F, Levy-Toledano S, Rosa JP, Bryckaert M. Regulation of c-jun-NH2 terminal kinase and extracellular-signal regulated kinase in human platelets. *Blood* 1999; 94: 3800–3805.

87 Torti M, Festetics ET, Bertoni A, Sinigaglia F, Balduini C. Clustering of integrin alphaIIb-beta3 differently regulates tyrosine phosphorylation of $pp72^{syk}$, $PLC\gamma2$ and $pp125^{FAK}$ in concanavalin A-stimulated platelets. *Thromb Haemost* 1999; 81: 124–130.

88 Parise LV. Integrin $\alpha_{IIb}\beta_3$ signalling in platelet adhesion and aggregation. *Curr Opin Cell Biol* 1999; 11: 597–601.

89 Brass LF, Shattil SJ. Identification and function of the high affinity binding sites for Ca^{2+} on the surface of platelets. *J Clin Invest* 1984; 73: 626–632.

90 Yamaguchi A, Yamamoto N, Kitagawa H, Tanoue, K, Yamazaki H. Ca^{2+} influx mediated through the GPIIb/IIIa complex during platelet activation. *FEBS Lett* 1987; 225: 228–232.

91 Bertolini G, Noris P, Spedini P, Balduini CL. Ristocetin-induced platelet agglutination stimulates GPIIb/IIIa-dependent calcium influx. *Thromb Haemost* 1995; 73: 689–692.

92 Francesconi M, Casonato A, Pontara E, Dalla Via L, Girolami A, Deana R. Type IIB von Willebrand factor induces phospholipase A_2 activation and cytosolic Ca^{2+} increase in platelets. *Biochem Biophys Res Commun* 1995; 214: 102–109.

93 Meijer EMY, Rosado JA, Heemskerk JWM, Sage SO. Store-

Cambridge Books Online © Cambridge University Press, 2009

mediated calcium entry is modulated by the occupancy of glycoprotein IIb/IIIa in human platelets. *J Physiol* 2000; 527: 77P-78P.

94 Hato T, Oda A, Ozaki Y et al. Outside-in signalling from integrin alpha IIb beta 3 into platelets in the absence of agonist-induced signalling. *Int J Hematol* 1997; 65: 385–395.

95 Ware JA, Decenzo MT, Smith M, Saitoh M. Calcium mobilization and glycoprotein IIb-IIIa complex ligands in epinephrine-stimulated platelets. *Am J Physiol* 1991; 260: H1619-H1624.

96 Nakamura T, Ariyoshi H, Kambayashi J et al. Effect of low concentration of epinephrine on human platelet aggregation analyzed by particle counting method and confocal microscopy. *J Lab Clin Med* 1991; 130: 262–270.

97 Saitoh M, Salzman EW, Smith M, Ware JA. Activation of protein kinase C in platelets by epinephrine and A23187: correlation with fibrinogen binding. *Blood* 1989; 74: 2001–2006.

98 Keularts IMLW, Van Gorp RMA, Feijge MAH, Vuist WMJ, Heemskerk JWM. α_{2A}-adrenergic receptor stimulation potentiates calcium release in platelets by modulating cAMP levels. *J Biol Chem* 2000; 275: 1763–1772.

99 Olbrich C, Aepfelbacher M, Siess W. Epinephrine potentiates calcium mobilization and activation of protein kinases in platelets stimulated by ADP through a mechanism unrelated to phospholipase C. *Cell Signal* 1989; 1: 483–492.

100 Alarayyed NA, Cooper MB, Prichard BN, Betteridge DJ, Smith CC. In vitro adrenaline and collagen-induced mobilization of platelet calcium and its inhibition by naftopidil, doxazosin and nifedipine. *Br J Clin Pharmacol* 1997; 43: 415–420.

101 Brass, L.F. Ca^{2+} homeostasis in unstimulated platelets. *J Biol Chem* 1984; 259: 12563–12570.

102 Rosado JA, Sage SO. Regulation of plasma membrane Ca^{2+} ATPase by small GTPases and phosphoinositides in human platelets. *J Biol Chem* 2000; 275: 19529–19535.

103 Heemskerk JWM, Vis P, Feijge MAH et al. Roles of phospholipase C and Ca^{2+}-ATPase in calcium responses of single, fibrinogen-bound platelets. *J Biol Chem* 1993; 268: 356–363.

104 Enouf J, Bredoux R, Papp B et al. Human platelets express the SERCA2b isoform of Ca^{2+}-transport ATPase. *Biochem J* 1992; 286: 135–140.

105 Bobe R, Bredoux R, Wuytack F et al. The rat platelet 97-kDa Ca^{2+}ATPase isoform is the sarcoendoplasmic reticulum Ca^{2+}ATPase 3 protein. *J Biol Chem* 1994; 269: 1417–1424.

106 Wuytack F, Papp B, Verboomen H et al. A sarco/endoplasmic reticulum Ca^{2+}-ATPase 3-type Ca^{2+} pump is expressed in platelets, in lymphoid cells, and in mast cells. *J Biol Chem* 1994; 269: 1410–1416.

107 Kovàcs T, Corvazier E, Papp B et al. Controlled proteolysis of Ca(2+)-ATPases in human platelet and non-muscle cell membrane vesicles. Evidence for a multi-sarco/endoplasmic reticulum Ca^{2+}-ATPase system. *J Biol Chem* 1994; 269: 6177–6184.

108 Martin V, Bredoux R, Corvazier E, Papp B, Enouf J. Platelet Ca^{2+} ATPases: a plural, species-specific, and multiple hypertension-regulated expression system. *Hypertension* 2000; 35: 91–102.

109 Papp B, Pàszty K, Kovàcs T et al. Characterization of the inositol trisphosphate-sensitive and insensitive calcium stores by selective inhibition of the endoplasmic reticulum-type calcium pump isoforms in isolated platelet membrane vesicles. *Cell Calcium* 1993; 14: 531–538.

110 Adunyah SE, Dean WL. Regulation of human platelet membrane Ca^{2+} transport by cAMP- and calmodulin-dependent phosphorylation. *Biochim Biophys Acta* 1987; 930: 401–409.

111 Johansson JS, Nied LE, Haynes DH. Cyclic AMP stimulates Ca^{2+}-ATPase-mediated Ca^{2+} extrusion from human platelets. *Biochim Biophys Acta* 1992; 1105: 19–28.

112 Lacabaratz-Porret C, Corvazier E, Kovacs T et al. Platelet sarco/endoplasmic reticulum Ca^{2+}ATPase isoform 3b and Rap 1b: interrelation and regulation in physiopathology. *Biochem J* 1998; 332: 173–181.

113 Pernollet MG, Lantoine F, Devynck MA. Nitric oxide inhibits ATP-dependent Ca^{2+} uptake into platelet membrane vesicles. *Biochem Biophys Res Commun* 1996; 222: 780–785.

114 Trepakova ES, Cohen RA, Bolotina VM. Nitric oxide inhibits capacitative cation influx in human platelets by promoting sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase-dependent refilling of Ca^{2+} stores. *Circ Res* 1999; 84: 201–209.

115 Kimura M, Aviv A, Reeves JP. K^+-dependent Na^+/Ca^{2+} exchange in human platelets. *J Biol Chem* 1993; 268: 6874–6877.

116 Kimura M, Jeanclos EM, Donnelly RJ, Lytton J, Reeves JP, Aviv A. Physiological and molecular characterization of the Na^+/Ca^{2+} exchanger in human platelets. *Am J Physiol* 1999; 277: H911-H917.

117 Brass LF. The effect of Na^+ on Ca^{2+} homeostasis in unstimulated platelets. *J Biol Chem* 1984; 259: 12571–12575.

118 Rink TJ, Sage SO. Stimulated calcium efflux from fura-2-loaded human platelets. *J Physiol* 1987; 393: 513–524.

119 Dean WL, Chen D, Brandt PC, Vanaman TC. Regulation of platelet plasma membrane Ca^{2+}-ATPase by cAMP-dependent and tyrosine phosphorylation. *J Biol Chem* 1997; 272: 15113–15119.

120 Choquette D, Hakim G, Filoteo AG, Plishker GA, Bostwick JR, Penniston JT. Regulation of plasma membrane Ca^{2+} ATPases by lipids of the phosphatidylinositol cycle. *Biochem Biophys Res Commun* 1984; 125: 908–915.

121 Wang KKW, Villalobo A, Roufogalis BD. The plasma membrane calcium pump: a multiregulated transporter. *Trends Cell Biol* 1992; 2: 46–51.

Cambridge Books Online © Cambridge University Press, 2009

18

Platelet signalling: protein kinase C

Charles S. Abrams and Marcelo G. Kazanietz

University of Pennsylvania, Philadelphia, USA

Protein kinase C, a family of serine-threonine kinases

Protein kinase C (PKC) isozymes are a family of kinases that play an essential role in the signal transduction mechanisms after activation of receptors. Although with a different pattern of isozyme expression, these phospholipid-dependent kinases are present in every single cell type, including platelets. PKC isozymes phosphorylate multiple cellular proteins in serine and threonine residues leading to a plethora of effects on cell proliferation and death, differentiation, morphology and adhesion[1,2]. In platelets, PKC isozymes control a variety of functions, including aggregation, release of granular contents, mobilization of intracellular calcium and regulation of cell shape. In addition, PKC isozymes play an important role in megakaryocyte differentiation.

PKC has been identified as the cellular receptor for the lipid second messenger diacylglycerol (DAG), and it is therefore a key enzyme in the signalling mechanisms after activation of receptors coupled to phospholipase C (PLC), a family of enzymes that leads to a transient elevation in membrane DAG levels. In addition, PKC isozymes are high affinity receptors for the phorbol ester tumour promoters, natural compounds which are the most common pharmacological activators of PKC both in vitro and in cellular systems. The higher potency of phorbol esters and their greater stability compared to the second messenger DAG make these compounds the preferred activators of PKC in experimental models. Because of the limitations of platelets as a system for genetic studies, pharmacological tools have been widely used in these cells. Both activation with phorbol esters (preferably with PMA or phorbol 12-myristate 13-acetate) and inhibition with specific PKC inhibitors have been the preferred approaches used to understand the involvement of PKC isozymes in platelet biology.

Table 18.1. PKC isoforms

Category	Isoform	Ca^{2+}-dependent?	DAG (or PMA)-responsive?	Present in platelets?
Classical	α	Yes	Yes	Yes
	β (βI & βII)	Yes	Yes	Yes
	γ	Yes	Yes	No
Novel	δ	No	Yes	Yes
	ε	No	Yes	Yes
	η	No	Yes	Yes
	θ	No	Yes	Yes
Atypical	λ	No	No	Yes, but low amounts
	ζ	No	No	Yes, but low amounts
Other	μ (aka PKD)	No	Yes	?
	ν	?	?	?

The PKC family comprises at least ten related kinases with differential expression in each cell type. PKC isozymes have been classified into three subclasses according to their structure and regulation: ‘classical’ (cPKCs), ‘novel’ (nPKCs) and ‘atypical’ (aPKCs). cPKCs comprise PKCα, βI, βII, and γ (Table 18.1) These PKC isozymes are calcium dependent. They can be activated not only by calcium but also by phorbol esters and DAG. The nPKCs (PKCδ, ε, η, and θ) are calcium insensitive but can be activated by phorbol esters and DAG. The third group (aPKCs) comprises PKCζ and λ. A unique characteristic of the aPKCs is that they do not bind phorbol esters or DAG even with low affinity, and therefore cannot be activated upon generation of DAG in the plasma membrane. A related enzyme, PKCμ or PKD, displays multiple unique features that makes it a distant relative of the PKC isozymes. Although some consider

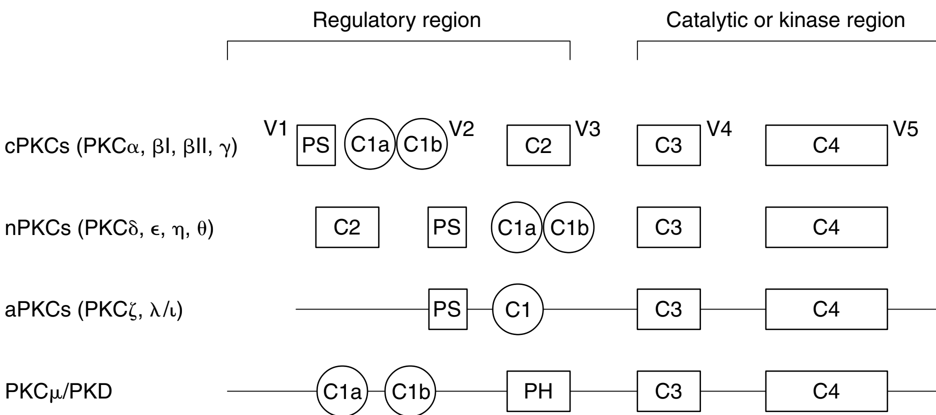


Fig. 18.1. Model showing the structure of PKC isoforms. Shown are the classical, novel, and atypical isoforms. Also shown is PKCμ/PKD.

PKCμ/PKD a member of the PKC family, this serine–threonine kinase has a unique pattern of substrate specificity that resembles that of calcium–calmodulin-dependent kinases. PKCμ/PKD is activated in cells by PKC phosphorylation and can therefore function downstream of PKCs. PKCν, a kinase related to PKCμ/PKD, has been recently cloned. No information exists on how this kinase is regulated in cells. Each PKC isozyme is the product of a separate gene, with the exception of PKCβI and βII, which are alternative spliced variants of the PKCβ gene[1–3].

All PKC isozymes are structurally related and have two well-defined domains: the N-terminal regulatory region and the C-terminal catalytic region (Fig. 18.1). The regulatory region possesses the motifs involved in the binding of the phospholipid cofactors, phorbol esters/DAG and calcium, and participates in protein–protein interactions that regulate PKC activity and localization. The C-terminal region is the kinase domain and includes motifs involved in ATP and substrate binding. A hinge region connects the regulatory and the catalytic domains. This flexible region is highly sensitive to proteolytic cleavage by cellular proteases. PKC isozymes possess domains that are highly conserved (C1 to C4) and variable domains (V1 to V5). The C1 domain in cPKCs and nPKCs is responsible for phorbol ester/DAG binding in membranes. Upon DAG binding, a conformational change relieves the autoinhibition conferred by the pseudosubstrate region located at the N-terminal region of PKCs. This autoinhibitory domain binds to the substrate binding site in the catalytic domain and keeps the enzyme in an inactive state in the absence of cofactors and activators. The C2 domain in cPKCs is involved in calcium and phospholipid binding, but only participates in phospholipid interactions in the calcium insensitive nPKCs. The C3 domain (or ATP binding site) and the C4 domain (or substrate binding site) present in the catalytic or kinase domain are essential for phosphotransferase activity[1,2].

Agonist-induced activation of PKC isozymes in platelets

One of the earliest responses of platelets to most agonists is the activation of PLC. Platelets contain β- and γ-forms of this enzyme. The β-forms are activated by G proteins, while the γ-forms (predominantly PLCγ_2) are regulated by tyrosine phosphorylation[4,5]. PLCβ is thought to be primarily responsible for the rapid burst of phosphoinositide hydrolysis that occurs during platelet activation by agonists such as thrombin and TxA_2 analogues. In general, PLCβ_1 and PLCβ_3 respond best to Gα, particularly members of the $G_q\alpha$ family, while PLCβ_2 responds best to Gβ_γ. Based upon studies with pertussis toxin and genetically modified mice, PAR-1 in platelets is thought to be coupled to PLCβ by Gα derived from G_q or G_{11}, as well as G$\beta\gamma$ derived from G_i[6–9]. TxA_2 receptors are coupled to PLC$\beta_{1/3}$ by Gα derived from G_q or G_{11}. Once activated, phospholipase C hydrolyses PI-4,5–P_2 to DAG plus IP_3[10–12]. DAG activates PKC and contributes to protein phosphorylation as will be discussed below[13,14]. IP_3 binds to receptors in the dense tubular system and releases sequestered Ca^{2+} [15–18]. Alternatively, atypical PKC isoforms have been shown to be activated by a PI3K-dependent pathway[19,20].

Platelets express multiple cPKCs, nPKCs and aPKCs. Although some discrepancies between different studies on PKC isozyme expression exist, it is clear that platelets express PKCα, β, δ, ε, η, and θ. PKCγ is not expressed in platelets. Although the presence of atypical PKCs has been

Cambridge Books Online © Cambridge University Press, 2009

detected in platelets, other studies reported very low levels for PKCζ and λ transcripts[21–24]. No information is available on the presence of PKCμ and PKCν in platelets.

cPKCs and nPKCs respond to phorbol esters by translocating from cytosol to membranes. Translocation of PKC isozymes is the hallmark for PKC activation and necessary for substrate phosphorylation. In platelets, PKC isozymes regulate multiple agonist-stimulated responses after activation of seven transmembrane receptors. Indeed, translocation of PKC isozymes in platelets occurs upon stimulation of numerous membrane receptors. For example, thrombin induces translocation of PKCα and PKCβ in human platelets. Translocation of PKC isozymes correlates with the increase in DAG levels in the plasma membrane observed upon stimulation with thrombin[25,26]. A stimulatory antibody (Mab.F11), which binds to the Fc gamma RII receptor and induces granular secretion and platelet aggregation, very potently translocates PKCα, β, δ, η, and θ. Interestingly, translocation of the DAG-unresponsive PKCζ also occurs, suggesting that other mechanisms in addition to DAG generation contribute to the subcellular redistribution of this aPKC[23].

As also reported in other cell types, PKC isozymes can get tyrosine phosphorylated in platelets. Studies in several cell types, including fibroblasts and keratinocytes, show that PKCδ is the main PKC isozyme that can be phosphorylated in tyrosine. Remarkably, platelet activation by thrombin induces tyrosine phosphorylation of PKCδ within 30 seconds. This leads to a 2–3 fold increase in kinase activity of this nPKC. The tyrosine phosphorylated form of PKCδ associates with a platelet particulate fraction (100,000 × g insoluble). This effect is not seen when platelets are activated by fibrinogen. Furthermore, $\alpha_{IIb}\beta_3$ receptor blockade did not affect the induction of PKCδ phosphorylation by thrombin, suggesting that the effect is independent of the $\alpha_{IIb}\beta_3$ pathway. The tyrosine kinase responsible for such phosphorylation upon thrombin receptor activation is not known. These results suggest that, in addition to the conventional PLC/DAG pathway, other regulatory mechanisms also take place in the control of PKC activity in platelets[27].

The role of PKC in platelet aggregation and secretion

The majority of the evidence that suggests a role for PKC in platelet activation is derived from studies that utilize pharmacologic activators, or inhibitors of this kinase. Although studies of this nature are subject to circumspection, certain themes do emerge from a large body of literature which use a diverse array of reagents. Collectively, these studies imply that PKC does contribute to both platelet aggregation and secretion. Genetic manipulation has created murine lines with null mutations in three different PKC isoforms (PKCβ, PKCγ and PKCε). As of yet there is no information whether these mutations induce a platelet phenotype[28–30].

Although seemingly contradictory, PKC may be involved in a variety of inhibitory responses in platelets. This hypothesis emerges from studies utilizing platelets which were preincubated with phorbol esters prior to the addition of an agonist. Examples of enzymes directly, or indirectly, inhibited by PKC include: PLC, adenylyl cyclase, phospholipase A2, phosphatidylinositol 3-kinase, and myosin light chain kinase[31–35]. This can lead to alterations in calcium release, cAMP production, and release of arachidonic acid. Several of the proteins that are directly phosphorylated by PKC during platelet activation have been identified, including the α subunits of the G proteins, G_z[36–38], G_{12} and G_{13}[39], myosin light chain (P20), pleckstrin (p47), actin binding protein[40], myosin light chain kinase and GP180, a transmembrane glycoprotein whose function is unknown[41]. Phosphorylation of Gzα appears to uncouple it from G$\beta\gamma$[42]. Phosphorylation of myosin light chain occurs at a site distinct from myosin light chain kinase[43] and is believed to affect the interaction of myosin with actin. Fig. 18.2 shows a model of these events.

Several reports have also indicated that PKC isozymes directly regulate receptor function in platelets. It is well known that, upon activation of seven-transmembrane receptors, PKC can phosphorylate these receptors leading to desensitization. An important example in platelets is the thromboxane receptor. Although a single thromboxane receptor gene has been identified, two splice variants have been cloned (TPα and TPβ). In human platelets, despite the presence of mRNA for both TP isoforms, isoform-specific antibodies against TP receptors detected only TPα. Upon activation of the TPα receptor with a TP agonist or arachidonic acid as a source of endogenous TxA_2, a rapid and transient phosphorylation of the receptor occurs. Pretreatment of platelets with the PKC inhibitor GF 109203X drastically reduced the phosphorylation of the receptor upon activation. Similarly, GF 109203X inhibits phorbol ester-induced phosphorylation of the receptor. PKC seems to phosphorylate the TPα receptor not only after activation with a TP agonist (homologous desensitization) but also after thrombin treatment (heterologous desensitization). The relative contribution of individual PKC isozymes to receptor desensitization mechanisms have yet to be determined[44,45].

Cambridge Books Online © Cambridge University Press, 2009

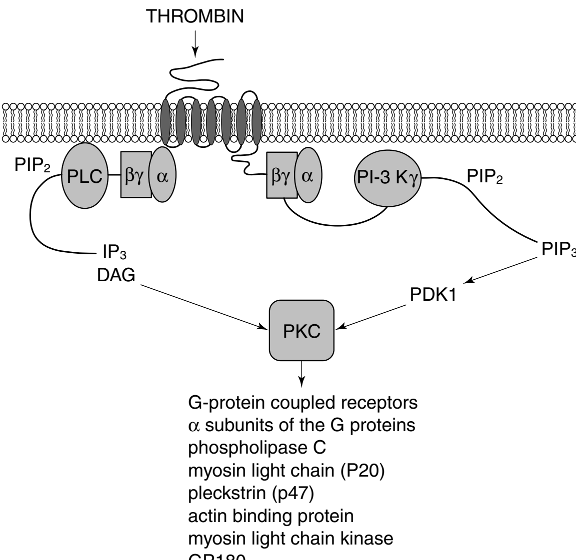


Fig. 18.2. Model showing the activation of different PKC isoforms. Stimulation of a pertussis toxin-insensitive G protein-coupled receptor, such as the thrombin receptor PAR1, stimulates $G\alpha$ subunit of heterotrimeric G-proteins. This can lead to the stimulation of PLCβ, and subsequently cPKCs and nPKCs. In contrast, stimulation of a pertussis toxin-sensitive G protein-coupled receptor releases free $G\beta\gamma$ heterodimers. This can also lead to the stimulation of PI3Kγ, and subsequently aPKC. This then enables PKC to phosphorylate platelet and megakaryocyte PKC substrates.

Platelet aggregation

The majority of agonists that lead to platelet aggregation also produce an increase in the activity of cPKCs and aPKCs. This association, along with the observation that phorbol esters induce platelet aggregation, suggests a potential role for PKC in platelet aggregation. Consistent with this hypothesis, inhibition of PKC by multiple different pharmacologic inhibitors prevents platelet aggregation in response to most platelet agonists. In addition, time-course experiments demonstrate that activation of PKC closely parallels the ability of αIIbβ3 to bind fibrinogen and support aggregation[46]. It is also notable that A.K. Rao and colleagues have described a patient with a bleeding disorder, who also has a defect in phosphorylation of platelet pleckstrin by PKC[47]. In addition, there is evidence that certain agents can potentiate subthreshold dosages of agonists through a PKC-dependent pathway ('PKC-priming')[48,49].

Which PKC isoform is required for platelet aggregation, and which signalling pathway is involved, is a subject of debate. Shattil and Brass have shown that a 'pseudo-substrate' like peptide which selectively inhibits cPKCs blocked activation of αIIbβ3[50]. This implies that cPKC isoforms contribute to 'inside-out' integrin signalling pathways in platelets. PKC may also contribute to 'outside-in' signalling pathways leading to actin assembly that are initiated after fibrinogen binds to αIIbβ3[51]. This will be discussed in the section on cytoskeletal changes.

Platelet secretion

Studies with PMA, a chemical stimulator of PKC, also suggest a role for this kinase in platelet secretion of both dense and alpha granules[52,53]. This appears to be independent of irreversible platelet aggregation. Further studies using stimulators of PKC such as PMA, mezerein, oleoylacetylglycerol, and (-)-indolactam V demonstrate that PKC can cause a calcium-dependent release of arachidonate[54]. In addition, multiple PKC isoform selective and non-selective inhibitors have been shown to block platelet secretion induced by several platelet agonists including thrombin[55,56].

The molecular mechanism by which PKC contributes to platelet secretion is just beginning to be elucidated. Agonist-mediated platelet secretion involves secretory pathways with homology to those found in other cells, including exocytosis in neurons[57]. These pathways involve the formation of SNARE complexes – a process facilitated by a platelet Sec1 protein called PSP. The ability of PSP to enhance SNARE complex formation is, in part, regulated by its phosphorylation by PKC. Although Rab proteins have been shown to play a role in vesicle trafficking in other cells, and several Rabs (isoforms 3b, 6, and 8) are phosphorylated by PKC, a direct connection between Rabs and platelet secretion has yet to be uncovered. There is some evidence that phosphorylation of MARCKS, an actin-associated protein, may also contribute to PKC-induced platelet secretion[58].

PKC, actin assembly, and pleckstrin

In platelets, PMA induces irreversible shape change and centripetal contractions[52,53,59]. Hartwig and coworkers have demonstrated that either thrombin-, or PMA-, induced stimulation of platelets leads to the assembly of F-actin, through a pathway that is independent of secretion[51]. This effect of PMA results in filopod extensions from the platelet surface. It appears to depend on PI3K since it coincides with the production of phospholipid products of this enzyme, and the actin effects are blocked by a chemical inhibitor of PI3K.

Cambridge Books Online © Cambridge University Press, 2009

The most visible substrate for PKC in platelets is p47 or pleckstrin, the 'Platelet and leukocyte C-kinase substrate'[60,61]. Pleckstrin is a 40–47 kDa hematopoietic protein which contains the two prototypic PH domains at its amino- and carboxyl-termini. Its phosphorylation has long been used as a marker for platelet activation. Though its function in vivo remains unclear, expressed pleckstrin can affect PIP_2-based signalling mediated by PLC, PI3K, and inositol phosphatases[35,62,63]. This may be mediated by the ability of pleckstrin to bind and sequester the substrate PIP_2. Ser^{113}, Thr^{114}, and Ser^{117}, the three residues phosphorylated by PKC, lie adjacent to, but not within, the amino-terminal PH domain, and phosphorylation at these sites has been shown to regulate the function of this PH domain[64]. A third functional motif has been described within pleckstrin[65]. This motif is termed the DEP domain, because of the first three proteins (Dishevelled, Egl-10 and pleckstrin) known to possess this sequence. The role of the DEP domain in pleckstrin, or other signalling proteins, is largely unknown.

Pleckstrin induces the formation of membrane ruffles

Based on the concept that PH domains recruit molecules to membranes, experiments were done to determine whether pleckstrin could bind to membranes and if so, whether this membrane attachment was mediated by the PH domains[66]. By cell fractionation it was determined that pleckstrin was associated with platelet membranes, and indirect immunofluorescence of transfected cells was used to answer the second part of this question. Overexpression of wild-type pleckstrin was able to induce the formation of membrane ruffles. Pleckstrin was associated with the plasma membranes and concentrated in the ruffles. By contrast, deletion of pleckstrin's amino terminal, but not its carboxyl terminal, PH domain created a pleckstrin variant unable to bind to membranes and alter its surface architecture. This argued that pleckstrin's effects on membranes was mediated by its amino- but not its carboxyl-terminal PH domain.

In order to evaluate the effect of phosphorylation on the ability of pleckstrin to induce membrane ruffle formation, two pleckstrin variants in which the sites of phosphorylation were mutated to either glycines or to glutamates were studied. The first variant is unable to be phosphorylated (non-phosphorylatable), while the second variant's charged residues mimic the state of phosphorylation (pseudo-phosphorylated)[64]. The pseudo-phosphorylated variant was fully able to associate with membranes and to induce ruffle formation. By contrast, the non-phosphorylatable variant was diffusely distributed and failed to lead to membrane ruffle formation, indicating that pleckstrin must be phosphorylated in order to attach to membranes and induce ruffle formation. Together, this implies that pleckstrin's ability to induce shape change is dependent on both its amino-terminal PH domains and is regulated by its phosphorylation sites.

Pleckstrin and actin organization

Based on the observations that pleckstrin could induce the formation of membrane ruffles, pleckstrin was analysed for its ability to cause reorganization of the actin cytoskeleton[67]. Experiments using fluorescently tagged phalloidin (a fungal toxin which binds and stains F-actin) demonstrated that pleckstrin-expressing Cos-1 cells had undergone cytoskeletal reorganization. Non-transfected cells had thick, central actin cables and an absence of actin around the periphery. Pleckstrin-expressing cells, by contrast, had no central actin cables and prominent cortical actin. Actin was found in small bundles underlying each of the membrane projections. Additional studies demonstrated that this effect on actin assembly was regulated by PKC phosphorylation of pleckstrin[67].

Small GTPases of the Rho family have been shown to play a critical role in cytoskeletal regulation[68]. The cortical actin polymerization initiated by G protein-coupled or growth factor receptors has been shown to require the action of Rac, a Rho family GTP-binding protein[69,70]. In order to determine if the actin reorganization induced by pleckstrin was similarly dependent on Rac, we coexpressed a myc-tagged, dominant negative variant of Rac (myc-V12N17 Rac), along with pleckstrin in Cos-1 cells and examined the pattern of phalloidin staining. We determined that coexpression of myc-V12N17 Rac was able to block many of the cytoskeletal effects of pleckstrin. Cells expressing the dominant negative Rac variant lacked cortical actin staining and dorsal projections. This argues that some of pleckstrin's effects on the actin cytoskeleton required the activity of Rac. By contrast, coexpression of a myc-tagged, dominant negative Cdc42 variant (myc-N17 Cdc42) had no effect on the pleckstrin-induced actin rearrangements. Cells expressing both pleckstrin and myc N17 Cdc42 remained with their cortical actin and small actin bundles intact and had no central actin cables. The data thus indicate that pleckstrin's effects on the actin cytoskeleton are at least partially dependent on the activation of Rac, but are independent of Cdc42.

Taken together, these studies have demonstrated a role for pleckstrin and its amino-terminal PH domain in reorganizing the actin cytoskeleton. Phosphorylation by PKC

Cambridge Books Online © Cambridge University Press, 2009

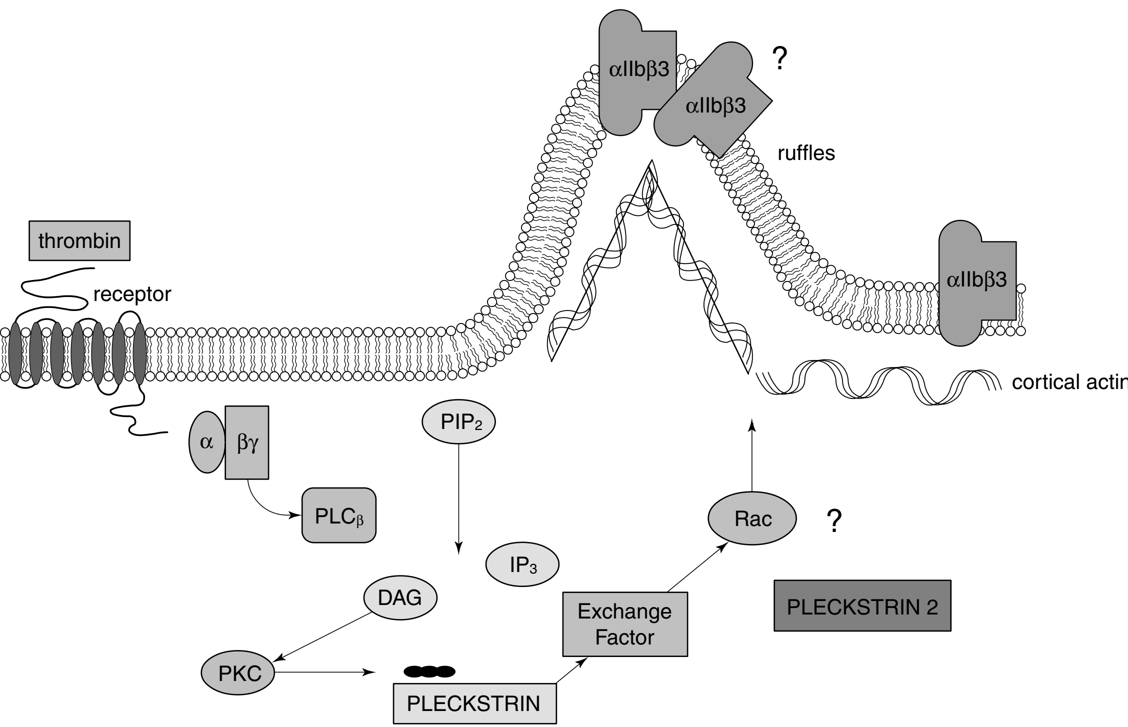


Fig. 18.3. Model showing the potential effect of pleckstrin on actin reorganization. Activation of PAR1, the predominant platelet thrombin receptor, leads to the stimulation of PLC*β*, and subsequently PKC, which phosphorylates pleckstrin. Presumably by a pathway dependent on stimulation of an exchange factor, this leads to activation of the small GTP-binding protein Rac. Stimulation of Rac then leads to some of the pleckstrin-induced cytoskeletal changes, including cortical actin and ruffle formation, through an integrin-dependent pathway.

at Ser^{113}, Thr^{114}, and Ser^{117} appears to be a regulatory mechanism for this function, perhaps by controlling the availability of sites for PIP_2-binding to the amino-terminal PH domain. Pleckstrin's effects on the cytoskeleton depend at least partially on Rac, but appear to be independent of Cdc42.

Pleckstrin and integrin-dependent cell spreading

Given the role of integrins in cytoskeletal organization and cell spreading, we investigated whether signalling from pleckstrin cooperated with signalling pathways involving the platelet integrin, αIIbβ3. Pleckstrin induced cell spreading in both transformed (Cos-1 and CHO) and non-transformed (REF52) cell lines, and this spreading was regulated by pleckstrin phosphorylation[71]. In REF52 cells, pleckstrin-induced spreading was matrix dependent, as evidenced by spreading of these cells on fibrinogen but not on fibronectin. Coexpression with αIIbβ3 did not enhance pleckstrin-mediated cell spreading in either REF52 or CHO cells. However, co-expression of the inactive variant αIIbβ3 Ser753Pro, or β3 Ser753Pro alone, completely blocked pleckstrin-induced spreading. This implies that αIIbβ3 Ser753Pro functions as a competitive inhibitor by blocking the effects of an endogenous receptor which is utilized in the signalling pathway involved in pleckstrin-induced cell spreading. Thus, expressed phosphorylated pleckstrin promotes cell spreading that is both matrix and integrin dependent. A model of the role of pleckstrin in these events is shown in Fig. 18.3.

Protein kinase C and megakaryocyte differentiation

Work from several laboratories has demonstrated a role for PKC isozymes in hematopoietic lineage commitment decisions. It is well established that the phorbol ester PMA promotes megakaryocyte differentiation. Experiments using HEL cells show that PMA causes a complete inhibition of proliferation and a marked increase in the surface expression of GP IIIa, a marker of megakaryocytic differentiation. These effects can be blocked by the selective PKC inhibitor GF 109203X[72]. Thrombopoietin (TPO), a major regulator of

Cambridge Books Online © Cambridge University Press, 2009

megakaryocytic lineage development transduces signals through activation of PKC. TPO induces the translocation of PKC from cytosol to membrane fractions. Western blot analysis revealed that PKCα and PKCβ translocate to the membranes upon TPO treatment. Although PKCα and PKCβ are involved in the mitogenic effect of TPO, PKC activation is not required for TPO-induced expression of megakaryocytic surface markers[73]. However guinea pig megakaryocyte spreading in culture is stimulated by PKC, and is blocked by the PKC inhibitors calphostin C and K5720[74].

Stimulation of PKC leads to the activation of the ERK (MAPK) signalling pathway, a cascade that plays an important role in proliferation and differentiation in many cell types. In K562 cells, sustained activation of the ERK (MAPK) cascade is required for megakaryocytic differentiation. PD09059, an inhibitor of MEK (the kinase that activates ERKs) completely abrogates megakaryocytic differentiation induced by PMA[75]. With the use of specific inhibitors for individual PKC isozymes, it was recently demonstrated that nPKCs but not cPKCs or aPKCs may be involved in the process of megakaryocytic differentiation. Using several different approaches, Racke et al.[76] showed that nPKCε may be a key transducer of signalling in differentiation. Moreover, constitutively active mutants of PKCε but not of other PKC isozymes strongly influence the function of GATA-1, a transcription factor known to be important in megakaryopoiesis and in the activation of the αIIb promoter[76].

References

1 Newton AC. Regulation of protein kinase C. *Curr Opin Cell Biol* 1997; 9: 161–167.
2 Ron D, Kazanietz MG. New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J* 1999; 13: 1658–1676.
3 Mellor H, Parker PJ. The extended protein kinase C superfamily. *Biochem J* 1998; 332: 281–292.
4 Cockcroft S, Thomas GMH. Inositol-lipid-specific phospholipase C isoenzymes and their differential regulation by receptors. *Biochem J* 1992; 288: 1–14.
5 Berridge MJ. Inositol trisphosphate and calcium signalling. *Nature* 1993; 361: 315–325.
6 Brass LF, Laposata M, Banga HS, Rittenhouse SE. Regulation of the phosphoinositide hydrolysis pathway in thrombin-stimulated platelets by a pertussis toxin-sensitive guanine nucleotide-binding protein. Evaluation of its contribution to platelet activation and comparisons with the adenylate cyclase inhibitory protein, G_i. *J Biol Chem* 1986; 261: 16838–16847.
7 Brass LF, Manning DR, Williams A, Woolkalis MJ, Poncz M. Receptor and G protein-mediated responses to thrombin in HEL cells. *J Biol Chem* 1991; 266: 958–965.
8 Offermanns S, Toombs CF, Hu YH, Simon MI. Defective platelet activation in G alpha(q)-deficient mice. *Nature* 1997; 389: 183–186.
9 Gabbeta J, Yang X, Kowalska MA, Sun L, Dhanasekaran N, Rao AK. Platelet signal transduction defect with Galpha subunit dysfunction and diminished Galphaq in a patient with abnormal platelet responses. *Proc Natl Acad Sci USA* 1997; 94: 8750–8755.
10 Billah MM, Lapetina EG, Cuatrecasas P. Phospholipase A2 and phospholipase C activity of platelets. *J Biol Chem* 1981; 255: 10227–10231.
11 Rittenhouse SE, Sasson JP. Mass changes in myoinositol trisphosphate in human platelets stimulated by thrombin. Inhibitory effects of phorbol ester. *J Biol Chem* 1985; 260: 8657–8660.
12 Lapetina EG. Inositol phospholipids and GTP-binding proteins in signal transduction in stimulated human platelets. *Adv Prostaglandin Thromboxane Leukotriene Res* 1986; 16: 217–224.
13 Lapetina EG, Reep B, Ganong BR, Bell RM. Exogenous sn-1,2-diacylglycerols containing saturated fatty acids function as bioregulators of protein kinase C in human platelets. *J Biol Chem* 1985; 260: 1358–1361.
14 Kaibuchi K, Takai Y, Sawamura M, Hoshijima M, Fujikura T, Nishizuka Y. Synergistic functions of protein phosphorylation and calcium mobilization in platelet activation. *J Biol Chem* 1983; 258: 6701–6704.
15 Adunyah S, Dean W. Inositol triphosphate-induced Ca^{2+} release from human platelet membranes. *Biochem Biophys Res Commun* 1985; 128: 1274–1280.
16 Authi KS, Crawford N. Inositol 1,4,5-trisphosphate-induced release of sequestered Ca^{2+} from highly purified human platelet intracellular membranes. *Biochem J* 1985; 230: 247–253.
17 O'Rourke FA, Halenda SP, Zavoico GB, Feinstein MB. Inositol 1,4,5-trisphosphate releases Ca^{2+} from a Ca^{2+}-transporting membrane vesicle fraction derived from human platelets. *J Biol Chem* 1985; 260: 956–962.
18 Brass LF, Joseph SK. A role for inositol triphosphate in intracellular Ca^{2+} mobilization and granule secretion in platelets. *J Biol Chem* 1985; 260: 15172–15179.
19 Nakanishi H, Brewer KA, Exton JH. Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 1993; 268: 13–16.
20 Toker A, Meyer M, Reddy KK et al. Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4–P2 and PtdIns-3,4,5–P3. *J Biol Chem* 1994; 269: 32358–32367.
21 Chang JD, Xu Y, Raychowdhury MK, Ware JA. Molecular cloning and expression of a cDNA encoding a novel isoenzyme of protein kinase C (nPKC). A new member of the nPKC family expressed in skeletal muscle, megakaryoblastic cells, and platelets. *J Biol Chem* 1993; 268: 14208–14214.

Cambridge Books Online © Cambridge University Press, 2009

22 Wang F, Naik UP, Ehrlich YH et al. A new protein kinase C, nPKCeta', and nPKCθ are expressed in human platelets: involvement of nPKCeta' and nPKCθ in signal transduction stimulated by PAF. *Biochem Biophys Res Commun* 1993; 191: 240–246.
23 Wang F, Naik UP, Ehrlich YH, Osada S, Ohno S, Kornecki E. Stimulatory antibody-induced activation and selective translocation of protein kinase C isoenzymes in human platelets. *Biochem J* 1995; 311: 401–406.
24 Oshevski S, Le Bousse-Kerdiles MC, Clay D et al. Differential expression of protein kinase C isoform transcripts in human hematopoietic progenitors undergoing differentiation. *Biochem Biophys Res Commun* 1999; 263: 603–609.
25 Baldassare JJ, Henderson PA, Burns D, Loomis C, Fisher GJ. Translocation of protein kinase C isozymes in thrombin-stimulated human platelets. Correlation with 1,2-diacylglycerol levels. *J Biol Chem* 1992; 267: 15585–15590.
26 Crabos M, Fabbro D, Stabel S, Erne P. Effect of tumour-promoting phorbol ester, thrombin and vasopressin on translocation of three distinct protein kinase C isoforms in human platelets and regulation by calcium. *Biochem J* 1992; 288: 891–896.
27 Moussazadeh M, Haimovich B. Protein kinase C-delta activation and tyrosine phosphorylation in platelets. *FEBS Lett* 1998; 438: 225–230.
28 Leitges M, Schmedt C, Guinamard R et al. Immunodeficiency in protein kinase cbeta-deficient mice. *Science* 1996; 273: 788–791.
29 Abeliovich A, Paylor R, Chen C, Kim JJ, Wehner JM, Tonegawa S. PKC gamma mutant mice exhibit mild deficits in spatial and contextual learning. *Cell* 1993; 75: 1263–1271.
30 Hodge CW, Mehmert KK, Kelley SP et al. Supersensitivity to allosteric GABA(A) receptor modulators and alcohol in mice lacking PKCepsilon. *Nat Neurosci* 1999; 2: 997–1002.
31 Bushfield M, Hopple SL, Gibson IF, Murdoch FA, MacIntyre DE. Effects of protein kinase C activation on human platelet cyclic AMP metabolism. *FEBS Lett* 1987; 222: 299–304.
32 Ryu SH, Kim U-H, Wahl MI et al. Feedback regulation of phospholipase C-β by protein kinase C. *J Biol Chem* 1990; 265: 17941–17945.
33 Iorio P, Gresele P, Stasi M et al. Protein kinase C inhibitors enhance G-protein induced phospholipase A2 activation in intact human platelets. *FEBS Lett* 1996; 381: 244–248.
34 Nucciarelli F, Gresele P, Nardicchi V et al. Evidence that cytosolic phospholipase A2 is down-regulated by protein kinase C in intact human platelets stimulated with fluoroaluminate. *FEBS Lett* 1999; 450: 39–43.
35 Abrams CS, Zhang J, Downes CP, Tang X-W, Zhao W, Rittenhouse SE. Phospho-pleckstrin inhibits $G_{\beta\gamma}$-activatable platelet phosphatidylinositol (4,5)P_2 3-kinase. *J Biol Chem* 1996; 271: 25192–25197.
36 Carlson K, Brass LF, Manning DR. Thrombin and phorbol esters cause the selective phosphorylation of a G protein other than G_i in human platelets. *J Biol Chem* 1989; 264: 13298–13305.
37 Lounsbury KM, Schlegel B, Poncz M, Brass LF, Manning DR. Analysis of $G_{z\alpha}$ by site-directed mutagenesis: sites and specificity of protein kinase C-dependent phosphorylation. *J Biol Chem* 1993; 268: 3494–3498.
38 Lounsbury KM, Casey PJ, Brass LF, Manning DR. Phosphorylation of G_z in human platelets: selectivity and site of modification. *J Biol Chem* 1991; 266: 22051–22056.
39 Offermanns S, Simon MI. $G\alpha_{15}$ and $G\alpha_{16}$ couple a wide variety of receptors to phospholipase C. *J Biol Chem* 1995; 270: 15175–15180.
40 Litchfield DW, Ball EH. Phosphorylation of caldesmon77 by protein kinase C in vitro and in intact human platelets. *J Biol Chem* 1987; 262: 8056–8060.
41 Bourguignon LYW, Walker G, Bourguignon GJ. Phorbol ester-induced phosphorylation of a transmembrane glycoprotein (GP180) in human blood platelets. *J Biol Chem* 1985; 260: 11775–11780.
42 Fields TA, Casey PJ. Phosphorylation of Gz alpha by protein kinase C blocks interaction with the beta gamma complex. *J Biol Chem* 1995; 270: 23119–23125.
43 Naka M, Nishikawa M, Adelstein RS, Hidaka H. Phorbol ester-induced activation of human platelets is associated with protein kinase C phosphorylation of myosin light chains. *Nature* 1983; 306: 490–492.
44 Habib A, Vezza R, Creminon C, Maclouf J, FitzGerald GA. Rapid, agonist-dependent phosphorylation in vivo of human thromboxane receptor isoforms. Minimal involvement of protein kinase C. *J Biol Chem* 1997; 272: 7191–7200.
45 Habib A, FitzGerald GA, Maclouf J. Phosphorylation of the thromboxane receptor alpha, the predominant isoform expressed in human platelets. *J Biol Chem* 1999; 274: 2645–2651.
46 Carty DJ, Spielberg F, Gear ARL. Thrombin causes subsecond changes in protein phosphorylation of platelets. *Blood* 1986; 67: 1738–1743.
47 Gabbeta J, Yang X, Sun L, McLane MA, Niewiarowski S, Rao AK. Abnormal inside-out signal transduction-dependent activation of glycoprotein IIb-IIIa in a patient with impaired pleckstrin phosphorylation. *Blood* 1996; 87: 1368–1376.
48 Vezza R, Roberti R, Nenci GG, Gresele P. Prostaglandin E2 potentiates platelet aggregation by priming protein kinase C. *Blood* 1993; 82: 2704–2713.
49 Ezumi Y, Uchiyama T, Takayama H. Thrombopoietin potentiates the protein-kinase-C-mediated activation of mitogen-activated protein kinase/ERK kinases and extracellular signal-regulated kinases in human platelets. *Eur J Biochem* 1998; 258: 976–985.
50 Shattil SJ, Cunningham M, Wiedmer T, Zhao J, Sims PJ, Brass LF. Regulation of GP IIb-IIIa receptor function studied with platelets permeabilized by the pore-forming complement proteins, C5b-9. *J Biol Chem* 1992; 267: 18424–18431.
51 Hartwig JH, Kung S, Kovacsovics T et al. D3 phosphoinositides and outside-in integrin signaling by glycoprotein IIb–IIIa mediate platelet actin assembly and filopodial extension induced by phorbol 12-myristate 13-acetate. *J Biol Chem* 1996; 271: 32986–32993.

Cambridge Books Online © Cambridge University Press, 2009

52 Zucker MB, Troll W, Belman S. The tumor-promoter phorbol ester (12–O-tetradecanoyl-phorbol-13-acetate), a potent aggregating agent for blood platelets. *J Cell Biol* 1974; 60: 325–336.

53 White JG, Rao GH, Estensen RD. Investigation of the release reaction in platelets exposed to phorbol myristate acetate. *Am J Pathol* 1974; 75: 301–314.

54 Halenda SP, Banga HS, Zavoico GB, Lau L-F, Feinstein MB. Synergistic release of arachidonic acid from platelets by activators of protein kinase C and Ca^{2+} ionophores. Evidence for the role of protein phosphorylation in the activation of phospholipase A_2 and independence from the Na^+/H^+ exchanger. *Biochemistry* 1989; 28: 7356–7363.

55 Geanacopoulos M, Turner J, Bowling KE, VandenBerg SR, Gear ARL. The role of protein kinase C in the initial events of platelet activation by thrombin assessed with a selective inhibitor. *Thromb Res* 1993; 69: 113–124.

56 Walker TR, Watson SP. Synergy between Ca^{2+} and protein kinase C is the major factor in determining the level of secretion from human platelets. *Biochem J* 1993; 289: 277–282.

57 Reed GL, Fitzgerald ML, Polgar J. Molecular mechanisms of platelet exocytosis: insights into the 'secret' life of thrombocytes. *Blood* 2000; 96: 3334–33342.

58 Elzagallaai A, Rose SD, Trifaro JM. Platelet secretion induced by phorbol esters stimulation is mediated through phosphorylation of MARCKS: a MARCKS-derived peptide blocks MARCKS phosphorylation and serotonin release without affecting pleckstrin phosphorylation. *Blood* 2000; 95: 894–902.

59 Schliwa M, Nakamura T, Porter KR, Euteneuer U. A tumor promoter induces rapid and coordinated reorganization of actin and vinculin in cultured cells. *J Cell Biol* 1984; 99: 1045–1059.

60 Imaoka T, Lynham JA, Haslam RJ. Purification and characterization of the 47,000-dalton protein phosphorylated during degranulation of human platelets. *J Biol Chem* 1983; 258: 11404–11414.

61 Haslam RJ, Lynham JA, Fox JEB. Effects of collagen, ionophore A23187 and prostaglandin E1 on the phosphorylation of specific proteins in blood platelets. *Biochem J* 1979; 178: 397–406.

62 Abrams CS, Wu H, Zhao W, Belmonte E, White D, Brass LF. Pleckstrin inhibits phosphoinositide hydrolysis initiated by G-protein-coupled and growth factor receptors. A role for pleckstrin's PH domains. *J Biol Chem* 1995; 270: 14485–14492.

63 Auethavekiat V, Abrams CS, Majerus PW. Phosphorylation of platelet pleckstrin activates inositol polyphosphate 5-phosphatase I. *J Biol Chem* 1997; 272: 1786–1790.

64 Abrams CS, Zhao W, Belmonte E, Brass LF. Protein kinase C regulates pleckstrin by phosphorylation of sites adjacent to the N-terminal pleckstrin homology domain. *J Biol Chem* 1995; 270: 23317–23321.

65 Ponting CP, Bork P. Pleckstrin's repeat performance: a novel domain in G-protein signaling? *Trends Biochem Sci* 1996; 21: 245–246.

66 Ma AD, Brass LF, Abrams CS. Pleckstrin associates with plasma membranes and induces the formation of membrane projections: requirements for phosphorylation and the NH2– terminal PH domain. *J Cell Biol* 1997; 136: 1071–1079.

67 Ma AD, Metjian A, Bagrodia S, Taylor S, Abrams CS. Cytoskeletal reorganization by G protein-coupled receptors is dependent on phosphoinositide 3-kinase gamma, a Rac guanosine exchange factor, and Rac. *Mol Cell Biol* 1998; 18: 4744–4751.

68 Mackay DJ, Hall A. Rho GTPases. *J Biol Chem* 1998; 273: 20685–20688.

69 Hartwig JH, Bokoch GM, Carpenter CL et al. Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. *Cell* 1995; 82: 643–653.

70 Azim AC, Barkalow K, Chou J, Hartwig JH. Activation of the small GTPases, rac and cdc42, after ligation of the platelet PAR-1 receptor. *Blood* 2000; 95: 959–964.

71 Roll RL, Bauman EM, Bennett JS, Abrams CS. Phosphorylated pleckstrin induces cell spreading via an integrin-dependent pathway. *J Cell Biol* 2000; 150: 1461–1466.

72 Hong Y, Martin JF, Vainchenker W, Erusalimsky JD. Inhibition of protein kinase C suppresses megakaryocytic differentiation and stimulates erythroid differentiation in HEL cells. *Blood* 1996; 87: 123–131.

73 Hong Y, Dumenil D, van der Loo B, Goncalves F, Vainchenker W, Erusalimsky JD. Protein kinase C mediates the mitogenic action of thrombopoietin in c-Mpl-expressing UT-7 cells. *Blood* 1998; 91: 813–822.

74 Leven RM. Differential regulation of integrin-mediated proplatelet formation and megakaryocyte spreading. *J Cell Physiol* 1995; 163: 597–607.

75 Racke FK, Lewandowska K, Goueli S, Goldfarb AN. Sustained activation of the extracellular signal-regulated kinase/ mitogen-activated protein kinase pathway is required for megakaryocytic differentiation of K562 cells. *J Biol Chem* 1997; 272: 23366–23370.

76 Racke FK, Wang D, Zaidi Z et al. A potential role for protein kinase C-ϵ in regulating megakaryocytic lineage commitment. *J Biol Chem* 2001; 276: 522–528.

Cambridge Books Online © Cambridge University Press, 2009

19

Platelet signalling: tyrosine kinases

Sylviane Levy-Toledano

INSERM U.348, IFR Circulation Lariboisière, Hôpital Lariboisière, Paris, France

Introduction

Protein phosphorylation plays a cardinal role in regulating many cellular processes in eukaryotes. In particular, protein phosphorylation is a major currency of signal transduction pathways. Processes that are reversibly controlled by protein phosphorylation require not only a protein kinase but also a protein phosphatase. Target proteins are phosphorylated at specific sites by one or more protein kinases and these phosphates are removed by specific protein phosphatases. In principle the extent of phosphorylation at a particular site can be regulated by changing the activity of the cognate protein kinase or protein phosphatase or both.

Protein tyrosine kinases (PTKs) catalyse the transfer of γ-phosphate of ATP to tyrosine residues of protein substrates and are critical components of signalling pathways that control a variety of biological responses including cell proliferation, migration, differentiation and survival. PTKs can be subvided in two large families: receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (NRTKs). Although platelets contain a small number of receptor tyrosine kinases on their surface, including the PDGF receptor[1,2], the majority of tyrosine kinases identified in platelets thus far are non-receptor forms, including members of the Src-kinase family, Syk kinase family, focal adhesion kinase (FAK) family, the Janus kinase (JAK) family and the Tec kinase family. Because of the key roles PTKs play in cellular signalling processes, their catalytic activity is tightly controlled in normal cells by protein tyrosine phosphatases, by other protein tyrosine or serine/threonine kinases and by autoregulatory mechanisms. The recent crystallographic structures of several members of both the RTK and NRTK families, together with extensive biochemical studies, afford an understanding at the molecular level of the autoregulation mechanisms to which PTKs are subject.

Src-family tyrosine kinases: members, structural features, and activation

The prototype member of the Src family protein tyrosine kinases was first identified as the transforming protein v-Src of the oncogenic retrovirus, Rous sarcoma virus[3]. c-Src is the cellular proto-oncogene of the v-Src oncogene. c-Src is expressed in a wide variety of tissues with a highest protein level detected in platelets, constituting 0.2–0.4 % of total platelet protein. Src kinase family includes at least eight members: Fyn, Lyn, Yes, Hck, Lck, Fgr, Blk and Yrk. Six of these have been identified in resting platelets: Fyn, Hck, Lyn, Yes and more recently Fgr and Lck[4,5]. Src, Fyn, Lck and Lyn are significantly tyrosine phosphorylated and all except Src are concentrated in detergent-insoluble fractions, suggesting a different subcellular localization of the Src-related kinases vs. Src.

Src protein kinases are 52–62 kDa proteins composed of distinct functional regions (Fig. 19.1). The N-terminal region, which includes a myristic acid moiety, is involved in targeting Src PTKs to cellular membranes. The highly conserved regulatory apparatus of the Src family members consists of two peptide binding modules, the Src-homology domains SH2 and SH3. The SH2 domain is approximately 100 amino acids long and binds to phosphorylated tyrosine residues in a sequence specific context. The specificity of individual SH2 domains lies in the 3–5 residues following the phosphotyrosine. Amino acids preceding phosphotyrosine may also be important for regulating binding affinity. The SH3 domain contains approximately 75 amino acids and binds to proline containing sequences. However, amino acids surrounding the prolines confer additional affinity and specificity for individual SH3 domains[6]. These modules, therefore mediate the formation of protein–protein interactions during signalling. Another critical structural feature of all Src-like kinases is the conserved C-terminal tyrosine residue which plays a

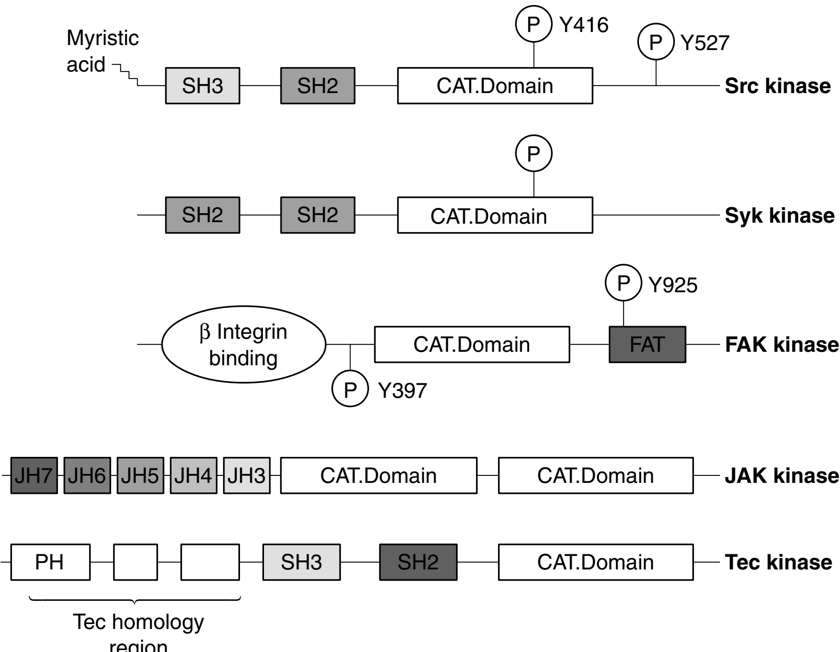


Fig. 19.1. Structural features of platelet non receptor tyrosine kinases. Linear diagrams of the different kinases illustrating their structural motifs as described in the text. CAT: catalytic domain, FAT: focal adhesion targeting domain, PH: pleckstrin domain.

critical role in regulating their catalytic activity. Phosphorylation of this C-terminal tyrosine (Tyr 527) is mediated by a distinct tyrosine kinase, Csk (c-Src kinase). The recent crystal structure of nearly full length Src has elucidated the role of the SH2 and SH3 domains in the autoregulation of Src family kinases[7]. Interactions between the SH2 domain and a C-terminal phosphotyrosine residue result in repression of catalytic activity, with additional inhibitory interactions provided by the SH3 domain. Dephosphorylation of the Tyr 527, or the binding of antibodies to the C-terminus, results in the catalytic activation of the Src-related kinases. The mechanisms by which the inhibitory phosphotyrosine residue is dephosphorylated and Src activation achieved have been investigated and suggest that the tyrosine phosphatase SHP1 would interact with Src and may serve by dephosphorylation of the COOH-terminal regulatory tyrosine[8,9]. The SH2-tail interaction is important for repression of Src activity because regulation is lost upon dephosphorylation of the phosphotyrosine. This dephosphorylation event is thought to be critical for Src activation by dissociating the C-terminal tail from the N-terminal SH2 domain, thereby allowing the autophosphorylation at another tyrosine residue (Tyr 416) located within the catalytic domain and required for catalytic activity (Fig. 19.2). The catalytic domain alone is functional as a tyrosine kinase but the SH2 and SH3 domains are required for full biological activity. Alternatively, Src can be activated by proteins containing proline-rich sequences that effectively compete with the SH2-kinase linker for binding to the SH3 domain, which again results in disruption of the inhibitory intra molecular constraints. Once released from the autoinhibited state, Src undergoes *trans*-autophosphorylation on a conserved tyrosine residue (Tyr 416) which stabilizes the active conformation[10] (Fig. 19.2).

Protein phosphorylation is regulated in large part by the subcellular localization of these kinases and their respective substrates. Stenberg et al.[11] reported that, unlike Src which is associated to plasma membranes, the other kinases colocalize with the coated vesicle protein, clathrin, confirming their association with this class of endocytotic vesicle.

Thrombin treatment of platelets causes an early integrin-independent activation of Src and Y527 dephosphorylation. However, the mechanisms by which Src is activated remain unclear. Very recently it has been described that Src tyrosine kinase is a novel direct effector of G proteins[12]. The authors demonstrate that Gαs and Gαi but neither Gαq, Gα12 nor G$\beta\gamma$, directly stimulate the kinase activity of down regulated c-Src by binding to the catalytic domain, changing the conformation of Src and leading to increased accessibility of the active site to sub-

Cambridge Books Online © Cambridge University Press, 2009

strates. If Src activation is uncoupled from integrin activation upon thrombin stimulation, it becomes associated with integrin complexes after platelet aggregation mediated by αIIb3. Therefore, there is a redistribution of activated c-Src to integrin dependent cytoskeletal complexes that allows accessibility of its SH2 and SH3 domains to other cellular proteins. One such protein is FAK, a protein tyrosine kinase that is phosphorylated and activated following engagement of αIIbβ3. FAK coprecipitates with Src and this coprecipitation is dependent on an intact FAK autophosphorylation site Y397 that binds to Src SH2 domains with a high affinity. It has been postulated that Src association with FAK may facilitate Src-mediated phosphorylation of other residues on FAK, some of which serve as binding sites for additional SH2 containing proteins. For example, phosphorylation of Y397 is required for phosphorylation of FAK on Y925, a Grb2-binding site and on Y576 and Y577 required for maximal kinase activity of FAK and for FAK: p130^{cas} complex formation. Indeed p130^{cas} and paxillin are phosphorylated by the FAK-Src complex enabling the recruitment of even more proteins[6]. c-Src also associates with the SH2-containing inositol 5-phosphatase, SHIP1, and is involved in its tyrosine phosphorylation downstream of αIIb3 integrin in human platelets[13]. SHIP1 may contribute to the phosphatidylinositol 3,4 bisphosphate (PI3,4 P2) accumulation, by dephosphorylation of phosphatidylinositol 3,4,5 trisphophate (PI 3,4,5 P3), playing a role in the strengthening of irreversible aggregation[14]. Once recruited and tyrosine phosphorylated, SHIP1 may also play a role as a docking protein allowing the formation of adequate signalling complexes at the proximity of integrin clusters.

The generation of mice bearing homologous disruption of Src genes develop severe osteopetrosis[15]. However, targeted disruption of the Src, Fyn, Yes and Lyn genes has revealed that the loss of a single Src family member does not perturb the hemostatic function of platelets. This would indicate that the presence of multiple Src kinases within the cell can compensate for the loss of an individual family member.

Syk tyrosine kinase

Syk, a 72 kDa tyrosine kinase, is expressed by all hematopoietic cells. It contains two N-proximal SH2 domains, two interdomain spacer regions, a catalytic domain and a C-terminal tail (Fig. 19.1). It is homologous to Zap 70 whose expression is limited to T-lymphocytes and NK cells. Syk is essential for lymphocyte development and for signal transduction via immune receptors in non-lymphoid cells.

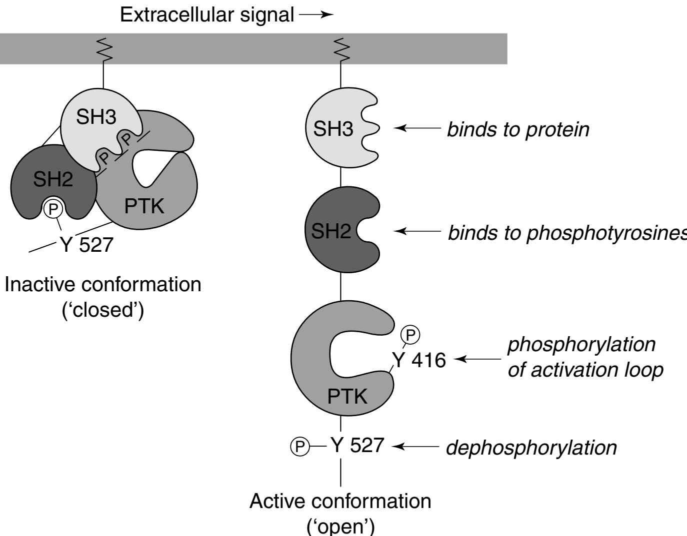


Fig. 19.2. Activation model of Src. Src kinases are found in either an active or inactive state depending on intramolecular associations. Interactions occur between the SH3 domain and polyproline helix II motif, and between the SH2 domain and the C-terminal phosphotyrosine residue. These associations which prevent activation of a Src kinase, are broken principally with the dephosphorylation of the C-terminal phosphotyrosine by a protein tyrosine phosphatase. This leads to an alteration of the three-dimensional structure of the molecule allowing the enzyme to assume an active conformation.

Although the extracellular domains of immune receptors are quite different, they share the ability to associate with invariant subunits which contain an immunoreceptor tyrosine-based activation motif (ITAM) in their cytoplasmic domains[16]. This motif is characterized by a consensus sequence that includes two tyrosines, typically 10–12 amino acids apart, which are rapidly phosphorylated following engagement of the immune receptor. A model for Syk activation has been proposed in lymphoid cells whereby the ligand-induced receptor clustering stimulates tyrosine phosphorylation of ITAMS by one or more Src family kinases resulting in Syk engagement through the tandem SH2 domains leading to Syk activation.

Syk has been identified in platelets and like Src family kinases it is rapidly activated following thrombin, ADP or collagen regardless of the activation and/or ligand binding status of the αIIbβ3 integrin. Syk activation correlates with its tyrosine phosphorylation; however, full activation of Syk requires integrin engagement[17]. The modes of Syk activation through immune response receptors and thrombin and integrin receptors are distinctly different. Syk has recently been implicated in collagen-induced platelet

Cambridge Books Online © Cambridge University Press, 2009

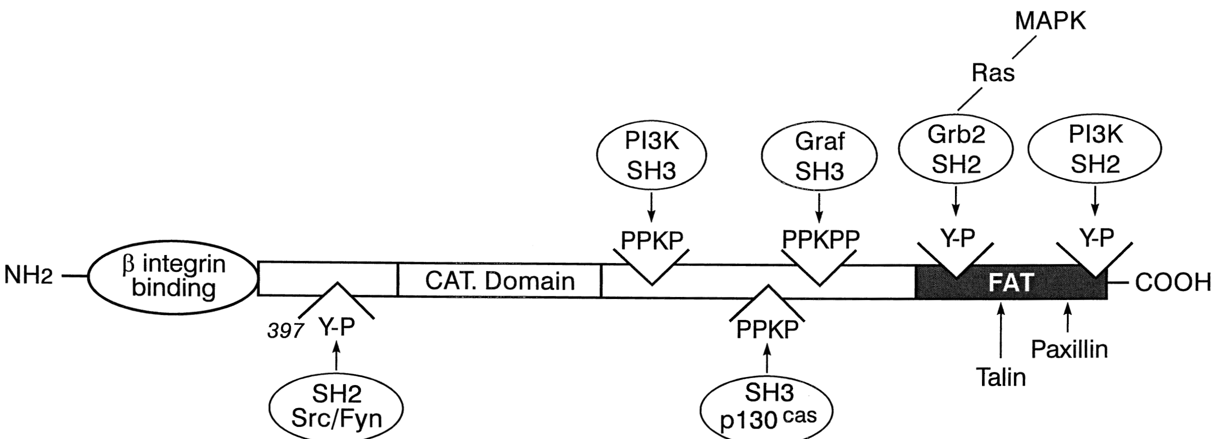


Fig. 19.3. Potential molecular interactions of FAK with cytoskeletal and signalling proteins. The phosphotyrosine residues serve as docking sites for SH2 containing signal transduction molecules such as Src/Fyn, PI3K, Grb2. The proline-rich sequences serve as docking sites for SH3 containing molecules such as p130cas, Graf and PI3K. Binding sites in the N and C terminal tails are required for FAK binding cytoskeletal proteins such as the unit of integrin, talin and paxillin.

signalling. Syk becomes tyrosine phosphorylated upon collagen-induced platelet activation and associates with GPVI through the ITAM containing Fcγ signalling subunit and Syk deficient murine platelets fail to respond to collagen[18].

Since the thrombin receptor and integrins do not possess an immuno receptor tyrosine-based activation motif, the mechanism of thrombin-induced activation of Syk remains unclear. Concerning the integrins, Gao et al.[19] demonstrated that Syk activation could be triggered by the binding of soluble fibrinogen to αIIb3 and that the cytoplasmic tails of both αIIb and β3 were needed for this activation since it was abolished by the truncation of αIIb or β3 tails. Moreover, Src or a related kinase appeared to be required for Syk activation by αIIbβ3 since a kinase inactive variant blocked αIIbβ3-induced Syk phosphorylation. Finally Sada et al.[20] present evidence that thrombin induces association of Syk with FAK in human platelets and this association is dependent upon the state of actin polymerization.

The identity of substrates and effectors of Syk is not well defined. Syk is reported to interact with and phosphorylate or activate several proteins including PLCγ[18], Vav (a guanine nucleotide exchange factor for small ras-like GTPases)[21], PI3 kinase[22] and an actin-binding protein cortactin[23,24]. Experiments conducted in Syk knockout mice indicate a critical role for this kinase in hemostasis as Syk $-/-$ mice suffer from severe hemorragic complications in utero and die within the perinatal period[25]. Experiments conducted in Syk deficient murine platelets, generated using targeting methodology, fail to respond to collagen.This would be due to the absence of phosphorylation of PLCγ leading to inhibition of PI4,5 P2 hydrolysis, absence of inositol 1,4,5 trisphosphate (IP3) synthesis, Ca^{2+} release and pleckstrin phosphorylation[18]. However, these same authors demonstrated the ability of Syk deficient platelets to aggregate in response to thrombin stimulation. It is conceivable that some other kinase can compensate for the lack of Syk in these platelets. Finally, no increase in tail bleeding time was observed when Syk $-/-$ animals were compared with Syk $+/-$ or Syk $+/+$ controls[18]. However, Syk deficiency was associated with rebleeding after primary hemostasis in two of five animals, indicating an unstable hemostatic plug formation, which can contribute to death due to hemorrhage that happens to most Syk knockout mice shortly after birth[25].

Focal adhesion kinase

Focal adhesion kinase has emerged as a primary mediator of integrin signalling. FAK is a non receptor tyrosine kinase of 125 kDa that colocalizes with integrins at sites at which a cell makes close adhesive contact with the extracellular matrix substratum[26,27]. Integrin-mediated cell adhesion promotes rapid FAK tyrosine phosphorylation, which results in the activation of signalling functions. Unlike the Src family tyrosine kinases, p125 FAK does not contain SH2 or SH3 domains. It has a catalytic domain flanked by large N and C terminal domains (Fig. 19.1). FAK has been shown to bind a variety of adapter and signalling molecules (Fig. 19.3). Six FAK tyrosines (397, 407, 576, 577, 861 and 925) have been identified as sites of adhesion dependent phos-

Cambridge Books Online © Cambridge University Press, 2009

phorylation. Tyr 397 is the only apparent site of autophosphorylation. Phosphorylation of Tyr 397 creates a high affinity binding site for the SH2 domains of Src-family tyrosine kinases including c-Src and Fyn, which could promote the activation by C-terminal tail displacement[28]. Phosphotyrosine 397 appears also to mediate interactions with SH2 domains of the p85 subunit of phosphatidylinositol 3-kinase (PI3K), which could subsequently stimulate its activity[29]. FAK can also bind to PI3K upon ligand engagement of integrin in thrombin-stimulated platelets. Guinebault et al.[30] have demonstrated that a GST fusion protein containing the p85 SH3 domain can bind to FAK as well as to a synthetic peptide derived from a proline-rich region of FAK, indicating a role of the SH3 domain of p85 in FAK association with PI3K. Phosphorylation of Tyr 576 and Tyr 577, which lie in the FAK kinase domain activation loop, appears to elevate FAK catalytic activity. Tyr 925 of FAK which is phosphorylated by Src in vitro has been identified as the binding site for the SH2 domain of Grb2[31]. Grb2 binding to phosphorylated FAK would be followed by recruitment of Sos and the activation of Ras/MAP kinase pathways. Recently, it has been reported that FAK can directly interact with PLCγ1. This interaction is mediated by the C-terminal SH2 domain of PLCγ1 binding to FAK phosphorylated Tyr 397[32]. FAK-null mice die in fetal life and, ex vivo, their fibroblasts form focal adhesions but migrate poorly[33].

Several groups have reported the identification of a second related FAK PTK referred to as RAFTK (related adhesion focal tyrosine kinase) also known as Pyk2 or CAK-β. RAFTK is related to FAK (48% identity, 65% similarity), which is known to play an important role in cell adhesion. RAFTK phosphorylation, unlike FAK phosphorylation occurs in the early phase of platelet activation, is not dependent on platelet aggregation and does not require integrin engagement[34]. Moreover, the activation of Pyk2 coincides with increased association with PI3K as determined by both anti-PI3K and anti Pyk2 immunoprecipitates. In addition, both increased PI3K activity and increased Pyk2 activity could be detected in immunoprecipitate following thrombin stimulation[35]. Finally, in immunoprecipitation studies, Pyk2 as well as FAK seems to associate with Shc through Grb2[36].

JAK family kinases

The JAK (JAnus Kinase or Just Another Kinase) family of kinases are a recently described group of signalling enzymes involved in cytokine and growth factor mediated signal transduction[37]. Four members of this family have been identified including JAK1, JAK2, JAK3 and TYK2 which share a high degree of structural similarity. The structure contains two C-terminal kinase related domains and five N-terminal other homology domains (JH3–JH7) (Fig. 19.1). Recently, it has been demonstrated that both JAK2 and TYK2 are present in human platelets. Signal transduction pathways activated by TPO, the ligand for c-Mpl, or thrombin, comprised the tyrosine phosphorylation of JAK2 and TYK2[38–41]. However, the time course of JAK2 phosphorylation in thrombin-stimulated platelets is slow, suggesting a potential role for this enzyme in the regulation of postaggregation responses.

Tec family kinases

Bruton's tyrosine kinase (77 kDa) (Btk) and Tec kinase (65 kDa) regulate the phosphorylation of PLCγ2 in B and T lymphocytes, respectively[42]. Btk is essential for normal B cell receptor signalling since the lack of expression of functional Btk in humans leads to the B-cell deficiency X-linked agammaglobulinemia (XLA). The structure of these enzymes shows that, in addition to containing an SH2 and an SH3 domain, the Tec family of kinases has two unique features: a pleckstrin (PH) domain and a Tec homology domain, which mediates association of Tec with Lyn[43] (Fig. 19.1). Btk and Tec kinase are also expressed in platelets and undergo phosphorylation in response to collagen-related peptide (CRP) and collagen. A role for Btk in platelets was recently demonstrated by the observation of decreased in vitro collagen-induced aggregation of platelets from XLA patients. It has been described that CRP and collagen, which bind to GPVI, induce Btk phosphorylation which plays a crucial role by regulating tyrosine phosphorylation and activation of PLCγ2[44].

However, since PLCγ2 tyrosine phosphorylation is only partially reduced in Btk-deficient platelets, there must be alternative pathways that regulate tyrosine phosphorylation of this enzyme. Other tyrosine kinases including Tec, a homologue of Btk, that is expressed in platelets[45] might partially substitute for Btk in regulating the tyrosine phosphorylation of PLCγ2 in XLA platelets[44,46]. Aggregation of Btk deficient platelets in response to low and high concentrations of thrombin was not significantly different from controls indicating that Btk is not essential for thrombin-mediated activation and that thrombin may selectively signal through Tec, the phosphorylation of which is highly dependent on platelet aggregation[46].

Cambridge Books Online © Cambridge University Press, 2009

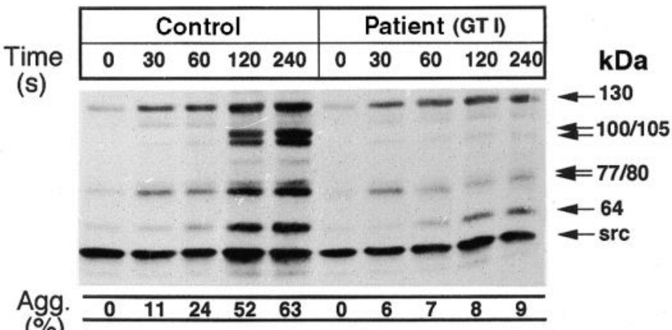


Fig. 19.4. Kinetics of thrombin-induced tyrosine phosphorylation of protein substrates in platelets from control and from type I Glanzmann thrombasthenia patient (GT I)[51].

Protein tyrosine phosphorylation events in human platelets

Platelet aggregation in response to a number of agonists is precipitated by an extremely rapid and complex set of signalling pathways. Initial signalling events activated by the G-protein coupled receptors, leads to stimulation of polyphosphoinositide turnover, arachidonic acid metabolism, calcium mobilization and influx and changes in protein phosphorylation on both serine/threonine and tyrosine residues. These events lead to activation of αIIbβ3, the major integrin receptor on platelets, by increasing its affinity/avidity for fibrinogen through a process known as inside-out signalling. In turn, ligand occupancy and clustering of αIIb3 trigger outside- in signalling that involves a rapid and dramatic increase in tyrosine phosphorylation of multiple proteins, influences cytoskeletal reorganization and culminates in platelet aggregation[47]. Towards the later stages of aggregation, tyrosine dephosphorylation occurs coincident with activation and/or redistribution of protein tyrosine phosphatases[19].

Inside–out signalling events

In the case of thrombin, which binds to heptahelical receptors coupled to heterotrimeric G proteins, one consequence important to inside-out signalling is activation of the PLCβ by the α subunit of Gq resulting in hydrolysis of PI4,5 P2 and production of second messengers, diacylglycerol and IP3. Mouse platelets that have been rendered null for Gq undergo shape change but fail to aggregate in response to thrombin, ADP or a thromboxane A2 receptor agonist[48]. Occupancy of many G protein-coupled receptors also leads to rapid activation of non-receptor protein tyrosine kinases including Src, Syk and Pyk2. This leads to tyrosine-phosphorylated proteins such as Vav (a guanine nucleotide exchange factor for the Rac GTPase)[49], PLCγ[50] and cortactin (a cortical actin-binding protein)[51]. Syk tyrosine phosphorylation takes place during the period when platelets are changing shape upon low concentrations of a thromboxane analogue, IBOP, and cortactin is associated with Syk[24]. This emphasizes the role of cortactin in cell morphological changes which involve cytoskeletal rearrangements. Phosphorylation of cortactin is also described in thrombin-stimulated thrombasthenic platelets, which do not aggregate, confirming the role of the actin-binding protein in the early steps of platelet activation independently of platelet aggregation[51] (Fig. 19.4).

Agonists binding to G protein coupled receptors also lead to activation of non-receptor tyrosine phosphatases including SHP1. Thrombin stimulation has been shown to induce very rapid increases in SHP1 tyrosine phosphorylation and association of SHP1 with Src[9], suggesting that SHP1 may serve to activate Src by dephosphorylating the COOH-terminal regulatory tyrosine.

Outside–in signalling

A dramatic increase in tyrosine phosphorylation is observed in platelets when fibrinogen binds to αIIbβ3 and promotes integrin clustering. Signalling is propagated by interactions between integrin cytoplasmic tails, signalling molecules and structural cytoskeletal proteins including vinculin, talin and α actinin. When platelets begin to form microscopic aggregates, there is detectable activation of c-Src. Furthermore studies in CHO cell transfectants indicate that fibrinogen binding can trigger activation of Syk due to combination of autophosphorylation and phosphorylation of Src[19]. Full aggregation is then associated with activation of the tyrosine kinase FAK which binds to the cytoplasmic tail of β3 integrin and results in an increased number of tyrosine phosphorylated substrates. These substrates include the 60 kDa band, Src, the 64 kDa band, the 77/80 kDa cortactin, the 100/105 kDa and 125/130 kDa doublets (Fig. 19.4). The first experimental evidence correlating platelet aggregation with platelet tyrosine phosphorylation came from the study of platelets from patients with Glanzmann thrombasthenia. This inherited platelet disorder, characterized by defective platelet aggregation consecutive to the failure of patient's platelets to bind fibrinogen after agonist stimulation, is due to a quantitative or qualitative defect in αIIbβ3 integrin. The tyrosine phosphorylation patterns of platelets from three thrombasthenic types differed significantly from the controls and showed identical alterations[51].

The most affected bands were the 100–105 kDa doublet

Cambridge Books Online © Cambridge University Press, 2009

and the 125 kDa (FAK), which were completely absent, confirming the key role of αIIbβ3 in protein phosphorylation of these substrates (Fig. 19.4). According to Izaguirre et al.[52], αIIbβ3 mediates tyrosine phosphorylation of a 105 kDa protein in activated platelets which would correspond to α actinin. The protease calpain[53], the phosphatases SHIP (an SH2 domain-containing inositol 5 phosphatase) and SHP1 (Src homology protein tyrosine phosphatase) and the non receptor protein tyrosine kinases Tec and FAK are among the proteins that become phosphorylated at this stage.

FAK provides a great number of potential molecular interactions with cytoskeletal and signalling proteins (Fig. 19.3). Autophosphorylation of FAK at Tyr 397 creates a binding site for Src through its SH2 domain. Ligand binding to integrin promotes the association of the Ras-activating complex Grb2–Sos with phosphotyrosine 925 on FAK and activation of MAP kinase. There is evidence that the association of FAK and PI3K is also regulated by tyrosine phosphorylation of FAK[29,54] leading to an increase in PI 3,4 P2 necessary to stabilize fibrinogen binding. The SH2 domain-containing SHIP is tyrosine phosphorylated and relocated to the cytoskeleton in an aggregation and integrin-dependent manner. This inositol phosphatase is known to dephosphorylate PI3,4,5 P3 and contributes to the accumulation of PI3,4 P2 shown to participate in the stabilization of platelet aggregates[55]. The localization of FAK to focal adhesions is, in turn, stabilized by its ability to associate with paxillin and talin[56].

It has been shown, recently, that outside-in signalling mediated by the αIIbβ3 involves the phosphorylation of tyrosine residues on the cytoplasmic tail of β3. Shc was the primary protein binding to phosphorylated β3, and transient Shc phosphorylation was observed when thrombin-stimulated platelets were allowed to aggregate[57]. It is possible that events downstream of Shc binding to phosphorylated β3 involve activation of the MAP kinase ERK 1/2 pathways.

Upon thrombin stimulation, the protein tyrosine phosphatase SHP1 was found to be tyrosine phosphorylated and translocated to the cytoskeleton after αIIbβ3 engagement[58]. SHP1 can therefore develop its tyrosine phosphatase activity, thereby modulating the signal propagation as described in erythropoietin (EPO)-mediated cell signalling. The association of SHP1 to the EPO receptor causes termination of proliferative signals, probably through receptor dephosphorylation or the dephosphorylation of downstream signalling molecules[59]. We do not know the precise role of SHP1 in platelet signalling. However, the importance of the protein tyrosine phosphatases in general is suggested by the powerful effects that result from the use of the inhibitor pervanadate. Previous data obtained in our laboratory[60] show that pervanadate induces platelet aggregation accompanied by generation of a huge amount of tyrosine-phosphorylated substrates. This would suggest that tyrosine phosphatase activity is absolutely necessary for platelets to avoid being activated.

Conclusions

Platelet tyrosine kinases play a critical role in coordinating, integrating and amplifying signals from multiple cell surface receptors. Many of them have been demonstrated to translocate to the cytoskeleton fraction of aggregated platelets.

The translocation and activation of FAK which binds to the integrin could represent one of the earliest signalling events induced by integrin occupancy on the cell surface due to FAK's numerous molecular interactions with signalling and cytoskeletal proteins. The formation of this protein network at focal adhesion sites is responsible for the irreversible platelet aggregation.

Despite considerable progress having been made in this area, many questions remain unsolved including the absence of knowledge of the effectors of the tyrosine kinases. A complete understanding of the substrates of these protein tyrosine kinases may allow us to better understand the role of tyrosine phosphorylation in regulating platelet function. Knockout mouse models as well as new pharmacological agents will help to define the molecular interactions and hierarchies between these enzymes.

References

1 Vassbotn FS, Havnen OK, Heldin CH, Holmsen H. Negative feedback regulation of human platelets via autocrine activation of the platelet-derived growth factor alpha receptor. *J Biol Chem* 1994; 269: 13874–13879.
2 Selheim F, Fukami M, Holmsen H, Vassbotn FS. Platelet-derived-growth-factor-induced signalling in human platelets: phosphoinositide-3-kinase-dependent inhibition of platelet activation. *Biochem J* 2000; 350: 469–475.
3 Brugge JS, Erikson RL. Identification of a transformation-specific antigen induced by an avian sarcoma virus. *Nature* 1977; 269: 346–349.
4 Pestina TI, Stenberg PE, Drucker BJ et al. Identification of the Src family kinases, Lck and Fgr in platelets. *Arterioscler Thromb Vasc Biol* 1997; 17: 3278–3285.
5 Corey SJ, Anderson SM. Src-related protein tyrosine kinases in hematopoiesis. *Blood* 1999; 93: 1–14.
6 Thomas SM, Brugge JS.Cellular functions regulated by Src family kinases. *Ann Rev Cell Dev Biol* 1997; 13: 513–609.

Cambridge Books Online © Cambridge University Press, 2009

7 Xu W, Harrison SC, Eck MJ. Three dimensional structure of the tyrosine kinase c-Src. *Nature* 1997; 385: 595–603.
8 Falet H, Ramos-Morales F, Bachelot C, Fischer S, Rendu F. Association of the protein tyrosine phosphatase PTP1C with the protein tyrosine kinase c-Src in human platelets. *FEBS Lett* 1996; 383: 169–169.
9 Somani AK, Bignon J, Mills G, Siminovitch KA, Branch DR. Src kinase activity is regulated by the SHP-1 protein tyrosine phosphatase. *J Biol Chem* 1997; 272: 21113–21119.
10 Hubbard SR, Mohammadi M, Schlessinger J. Autoregulatory mechanisms in protein tyrosine kinases. *J Biol Chem* 1998; 273: 11987–11990.
11 Stenberg PE, Pestina TI, Barrie RJ, Jackson CW. The Src family kinases Fgr, Fyn, Lck and Lyn, colocalize with coated membranes in platelets. *Blood* 1997; 89: 2384–2393.
12 Yong-Chao M, Huang J, Ali S, Lowry W, Huang XY. Src tyrosine kinase is a novel direct effector of G proteins. *Cell* 2000; 102: 635–646.
13 Giuriato S, Bodin S, Erneux C et al. pp60c-Src associates with the SH2-containing inositol-5-phosphatase SHIP1 and is involved in its tyrosine phosphorylation downstream of αIIb/β3 integrin in human platelets. *Biochem J* 2000; 348: 107–112.
14 Kovasovics T, Bachelot C, Toker A et al. Phosphoinositide 3-kinase inhibition spares actin assembly in activating platelets but reverses platelet aggregation. *J Biol Chem* 1995; 270: 11358–11366.
15 Soriano P, Montgomery C, Gesk R, Bradley A.Targeted disruption of c-Src protoncogene leads to osteopetrosis in mice. *Cell* 1991; 64: 693–702.
16 Turner M, Schwerghoffer E, Colucci F, Di Santo JP, Tybulewicz VL.Tyrosine kinase Syk: essential functions for immuno receptor signalling. *Immunol Today* 2000; 21: 148–154.
17 Law DA, Nannizi-Alaimo L, Ministri K et al. Genetic and pharmacological analyses of Syk function in αIIbβ3 signalling in platelets. *Blood* 1999; 93: 2645–2652.
18 Poole A, Gibbins JM, Turner M et al. The Fc receptor gamma-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *EMBO J* 1997; 16: 2333–2338.
19 Gao J, Zoller KE, Ginsberg MH, Brugge JS, Shattil SJ. Regulation of the pp72 Syk protein tyrosine kinase by platelet integrin αIIbβ3. *EMBO J* 1997; 16: 6414–6425.
20 Sada K, Minami Y,Yamamura H. Relocation of Syk protein tyrosine kinase to the actin filament network and subsequent association with FAK. *Eur J Biochem* 1997; 248: 827–833.
21 Teramoto H, Salem P, Robbins KC, Bustelo GR, Gutkind JS. Tyrosine phosphorylation of the Vav protooncogene product links FcγRI to the Rac-JNK pathway. *J Biol Chem* 1997; 272: 10751–10755.
22 Yanafi S, Sada K, Tohyana Y et al. Translocation, activation and association of protein tyrosine kinase (p72 Syk) with phosphatidylinositol 3-kinase are early events during platelet activation. *Eur J Biochem* 1994; 224: 329–333.
23 Maruyama S, Kurosaki T, Sada K, Yamanashi Y, Yamamoto T, Yamamura H. Physical and functional association of cortactin with Syk in human leukemic cell lines K 562. *J Biol Chem* 1997; 271: 6631–6635.
24 Gallet C, Rosa JP, Habib A, Lebret M, Levy-Toledano S, Maclouf J. Tyrosine phosphorylation of cortactin associated with Syk accompanies thromboxane analogue-induced platelet shape change. *J Biol Chem* 1999; 274: 23610–23616.
25 Turner M, Mee JP, Costello PS et al. Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature* 1995; 378: 278–285.
26 Schaller MD, Parsons JT.Focal adhesion kinase and associated proteins. *Curr Opin. Cell Biol* 1994; 6: 705–710.
27 Schaller MD, Otey CA, Hildebrand JD, Parsons JT. Focal adhesion kinase and paxillin bind to peptides mimicking β integrin cytoplasmic domains. *J Cell Biol* 1995; 130: 1181–1187.
28 Eide BL, Turck CW, Escobedo JA. Identification of Tyr-397 as the primary site of tyrosine phosphorylation and pp60 Src association in the focal adhesion kinase pp125 FAK. *Mol Cell Biol* 1995; 15: 2819–2827.
29 Chen HC, Appeddu PA, Isoda H, Guan JL. Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. *J Biol Chem* 1996; 271: 26329–26334.
30 Guinebaut C, Payrastre B, Racaud-Sultan C et al. Integrin-dependent translocation of PI3-kinase to the cytoskeleton of thrombin-activated platelets involves specific interactions of p85α with actin filaments and focal adhesion kinase. *J Cell Biol* 1995; 129: 831–842.
31 Schlaepfer DD, Hanks SK, Hunter T, vander Geer P. Integrin-mediated signal transduction linked to Ras pathway by Grb2 binding to focal adhesion kinase. *Nature* 1994; 372: 786–791.
32 Zhang X, Chattopadhyah A, Ji QS et al. Focal adhesion kinase promotes phospholipase C-γ1 activity. *Proc Natl Acad Sci USA* 1999; 96: 9021– 9026.
33 Ilic D, Furata Y, Kanazawa S et al. Reduced cell motility and enhanced focal adhesion formation in cells from FAK-deficient mice. *Nature* 1995; 377: 539–544.
34 Raja S, Avraham S, Avraham H. Tyrosine phosphorylation of the novel protein-tyrosine kinase RAFTK during an early phase of platelet activation by an integrin glycoprotein IIb-IIIa-independent mechanism. *J Biol Chem* 1997; 272: 10941–10947.
35 Sayed MR, Sheid MP, Stevens CM, Duronio V. Thrombin-stimulated phosphatidylinositol 3-kinase activity in platelets is associated with activation of PYK2 tyrosine kinase: activation of both enzymes is aggregation independent. *J Cell Physiol* 2000; 183: 314–320.
36 Ohmori T, Yatomi Y, Asazuma N, Satoh K, Ozaki Y. Involvement of proline-rich tyrosine kinase 2 in platelet activation: tyrosine phosphorylation mostly dependent on alpha IIb beta 3 integrin and protein kinase C, translocation to the cytoskeleton and association with Shc through Grb2. *J Biol Chem* 2000; 347: 561–569.
37 Wilks AF, Harpur AG, Kurban RR, Ralph SJ, Zurcher G, Ziemecki A. Cytokine signal transduction and the JAK family of protein tyrosine kinases. *Bioassays* 1994; 16: 313–320.
38 Miyakawa Y, Oda A, Drucker BJ et al. Recombinant thrombo-

Cambridge Books Online © Cambridge University Press, 2009

poietin induces rapid tyrosine phosphorylation of Janus kinase 2 and Shc in human blood platelets. *Blood* 1995; 6: 23–27.

39 Rodriguez-Linares B, Watson SP. Phosphorylation of JAK2 in thrombin-stimulated human platelets. *FEBS Lett* 1994; 352: 335–338.

40 Rodriguez-Linares B, Watson SP. Thrombopoietin potentiates activation of human platelets in association with JAK2 and TYK2 phosphorylation. *Biochem J* 1996; 316: 93–98.

41 Fontenay-Roupie M, Huret G, Loza JP et al. Thrombopoietin activates human platelets and induces tyrosine phosphorylation of p80/85 cortactin. *Thromb Haemost* 1998; 79: 195–201.

42 Schaeffer EM, Schwartzberg PL. Tec family kinases in lymphocyte signalling and function. *Curr Opin Immunol* 2000; 12: 282–288.

43 Mano H, Yamashita Y, Miyazato A, Miura Y, Ozawa K.Tec protein tyrosine kinase is an effector molecule of Lyn protein-tyrosine kinase. *FASEB J* 1996; 10: 637–641.

44 Quek LS, Bolen J, Watson SP. A role for Bruton's tyrosine kinase (Btk) in platelet activation by collagen. *Curr Biol* 1998; 8: 1137–1140.

45 Laffargue M, Ragab-Thomas JM, Ragab A. Phosphoinositide3-kinase and integrin signalling are involved in activation of Bruton's tyrosine kinase in thrombin stimulated platelets. *FEBS Lett* 1999; 443: 66–72.

46 Oda A, Ikeda Y, Ochs HD et al. Rapid tyrosine phosphorylation and activation of Bruton's tyrosine/Tec kinases in platelets induced by collagen binding or CD32 cross-linking. *Blood* 2000; 95: 1663–1670.

47 Shatill SJ, Kashiwagi H, Pampori N. Integrin signalling: the platelet paradigm. *Blood* 1998; 91: 2645–2667.

48 Offermans S, Toombs CF, Hu YH, Simon MI. Defective platelet activation in Gαq-deficient mice. *Nature* 1997; 389: 183–186.

49 Cichowski K, Brugge JS, Brass LF. Thrombin receptor activation and integrin engagement stimulate tyrosine phosphorylation of the proto-oncogene product, p95vav, in platelets. *J Biol Chem* 1996; 271: 7544–7549.

50 Tate BF, Rittenhouse SE. Thrombin activation of human platelets causes tyrosine phosphorylation of PLCγ2. *Biochim Biophys Acta Mol Cell Res* 1993; 1178: 281–285.

51 Rosa JP, Artçanuthurry V, Grelac F, Maclouf J, Caen JP, Levy-Toledano S. Reassessment of protein tyrosine phosphorylation in thrombasthenic platelets. Evidence that phosphorylation of cortactin and a 64 kDa protein is dependent on thrombin activation and integrin αIIbβ3. *Blood* 1997; 89: 4385–4390.

52 Izaguirre G, Aguirre L, Ji P, Aneskievich B, Haimovich B.Tyrosine phosphorylation of alpha-actinin in activated platelets. *J Biol Chem* 1999; 274: 37012–37020.

53 Frangioni JV, Oda A, Smith M, Salzman EW, Neel BL. Calpain-catalysed cleavage and subcellular relocation of protein phosphotyrosine phosphatase (PTP-1B) in human platelets. *EMBO J* 1993; 12: 4843–4856.

54 Bachelot C, Rameh L, Parsons JT, Cantley LC. Association of phosphatidylinositol 3-kinase, via the SH2 domains of p85 with focal adhesion kinase in polyoma middle T-transformed fibroblasts. *Biochim Biophys Acta* 1996; 1311: 45–52.

55 Giuriato S, Payrastre B, Drayer L et al. Tyrosine phosphorylation and relocation of SHIP are integrin mediated in thrombin-stimulated human platelets. *J Biol Chem* 1997; 272: 26857–26863.

56 Parsons JT. Integrin-mediated signalling regulation by protein tyrosine kinases and small GTP-binding proteins. *Curr Opin Cell Biol* 1996; 8: 146–152.

57 Cowan JK, Law D, Phillips D. Identification of Shc as the primary protein binding to the tyrosine phosphorylated β3 subunit of αIIbβ3 during outside-in integrin platelet signalling. *J Biol Chem* 2000; 275: 36423–36429.

58 Li RY, Gaits F, Ragab-Thomas JM et al. Protein tyrosine phosphatase SHP-1 fails to associate with cytoskeleton but is normally phosphorylated upon thrombin stimulation of thrombasthenic platelets. *Thromb Haemost* 1997; 77: 150–154.

59 Klingmuller U, Lorenz O, Cantley LC, Neel BG, Lodish HF. Specific recruitment of SHP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. *Cell* 1995; 80: 729–738.

60 Levy-Toledano S, Gallet C, Nadal F, Bryckaert M, Maclouf J, Rosa JP. Phosphorylation and dephosphorylation mechanisms in platelet function. A tightly regulated balance. *Thromb Haemost* 1997; 78: 226–233.

Cambridge Books Online © Cambridge University Press, 2009

20

Platelet signalling: cAMP and cGMP

James L. Daniel[1], Barrie Ashby[1] and Fabio M. Pulcinelli[2]

[1]*Department of Pharmacology and Sol Sherry Thrombosis Research Center, Temple University Medical School Philadelphia, USA*
[2]*Department of Experimental Medicine and Pathology, University La Sapienza, Rome, Italy*

History

The first report that cyclic AMP had an effect on platelets came from Marcus and Zucker[1], who showed that dibutiryl cyclic AMP, a cell permeant cyclic AMP analogue, inhibited platelet aggregation. Ardlie et al.[2] showed that methyl xanthines, which inhibit cyclic AMP phosphodiesterase and hence elevate cyclic AMP levels, also inhibit platelet aggregation and proposed that cyclic AMP is an important mediator of platelet function. In 1969, several groups of investigators reported that the level of platelet cyclic AMP regulated platelet function and showed that both platelet agonists and antagonists could affect the intraplatelet concentration of cyclic AMP[3–6]. Salzman suggested that cyclic AMP might be the ultimate mediator of platelet activation in that agents that inhibited aggregation increased cyclic AMP, while agents that activated platelets did so by lowering the basal level of cyclic AMP[7]. However, the idea that agonists activate platelets by lowering basal cyclic AMP was convincingly refuted by Haslam et al.[8].

Elevated levels of cyclic GMP also inhibit platelet activation[9,10] and platelet activators elevate cyclic GMP[11]. However, the latter effect has been shown to be a consequence of aggregation and does not occur during other forms of platelet activation[12]. A schematic summary of the information in this review is shown in Fig. 20.1.

Platelet receptors coupled to adenylyl cyclase activation

Prostaglandins that elevate cyclic AMP levels are potent inhibitors of platelet activation. Adenosine acts through the A2A receptor to activate adenylyl cyclase. The rank order of potency of prostaglandins as inhibitors of human platelet activation is PGI2 > 6 keto-PGE1 > PGE1 > PGD2 > 6-ketoPGF2α > PGE2 > PGF2α. The order reflects the importance of the prostacyclin (IP) receptor in mediating prostaglandin effects on platelet adenylyl cyclase. There are three distinct receptors for PGE2, and separate receptors for PGD2 and PGF2α. Table 20.1 shows a summary of the platelet receptors coupled to adenylyl cyclase.

IP receptor

The IP receptor mediates the action of PGI2. PGE1 also binds to the IP receptor with relatively high affinity. The IP receptor is linked to stimulation of adenylyl cyclase through the G protein Gs. Cloning and expression of the receptor confirmed that the receptor also couples to Ca^{2+} mobilization. The IP receptor is desensitized by protein kinase C but not by protein kinase A[13]. Studies on isolated platelets show that the IP receptor is also susceptible to down-regulation[14].

EP2 receptor

The EP2 receptor was defined when relaxant and contractile effects of PGE2 on guinea pig trachea smooth muscle were separated using the selective EP1 antagonists, SC 19220 and AH 8609[15]. The selective EP2 agonist butaprost has also proved useful in functional characterization of the receptor. The EP2 receptor couples to an increase in cyclic AMP. However, recent identification of the EP4 receptor, which also couples to an increase of cyclic AMP formation, suggests that some of the functions of PGE2 may not be mediated by EP2. The recombinant EP2 receptor is resist-

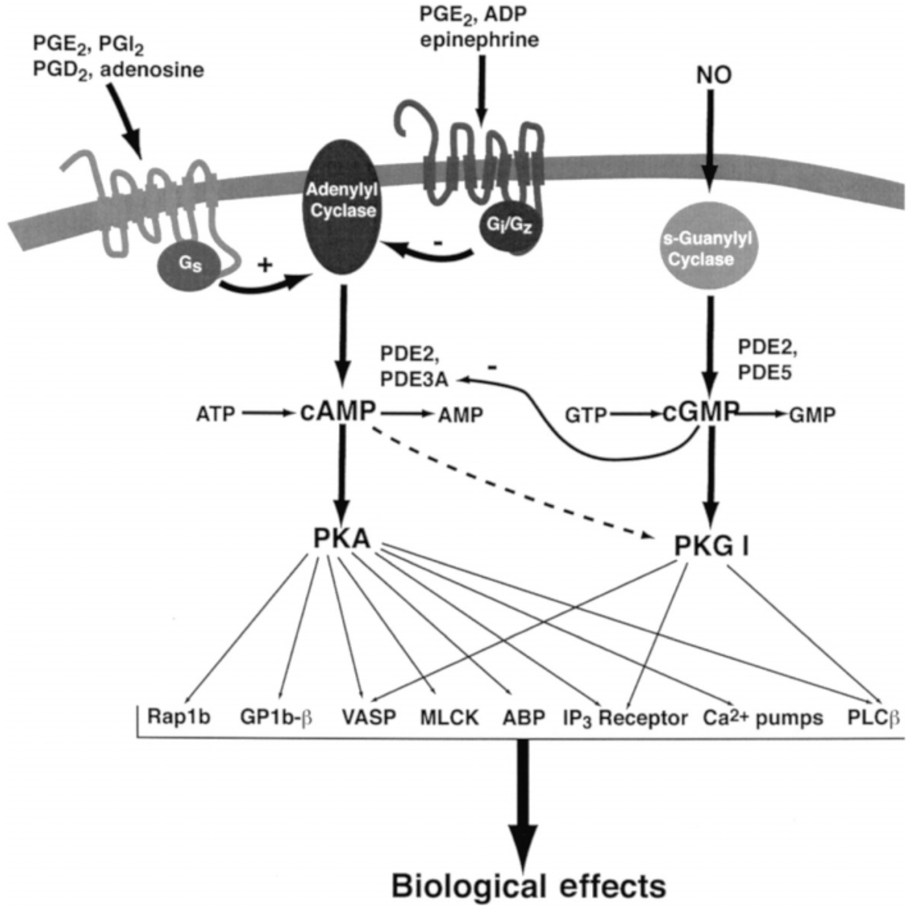


Fig. 20.1. A schematic summary of the receptors coupled to cAMP and cGMP and downstream effectors of cyclic nucleotides in platelets.

Table 20.1. Platelet receptors coupled to adenylyl cyclase regulation

Receptor name	Principal agonists	Selective agonists	Antagonists	adenylyl cyclase regulation
IP	PGI2	Iloprost		Positively
EP2	PGE1, PGE2	Butaprost	SC 19220, AH 8609	Positively
EP4	PGE1, PGE2		AH 23848B	Positively
DP	PGD2	9-deoxy-D^9-PGD2	BWA868C	Positively
A2A	Adenosine	CGS 21680	ZM241385	Positively
EP3	PGE2	Sulprostone, enprostil		Negatively
P2Y12	ADP		AR-C66096, thienopyridine	Negatively
α2a-adrenergic	Epinephrine, norepinephrine	Clonidine	Yohimbine	Negatively

Cambridge Books Online © Cambridge University Press, 2009

ant to agonist-induced short-term desensitization but undergoes down-regulation in response to long-term desensitization. Because PGE2 can cause an increase in cyclic AMP and inhibit platelet function, platelets almost certainly possess either the EP2 or the EP4 receptor.

EP4 receptor

The EP4 receptor couples to an increase in cyclic AMP, similar to the EP2 receptor. There are no selective agonists for the EP4 receptor; the EP2 selective agonist butaprost is not active at the EP4 receptor. Actions of PGE2 at the EP4 receptor can be assigned by comparison of butaprost and other EP receptor agonists. The receptor has a long carboxy terminal domain containing numerous serine and threonine residues. The EP4 receptor is susceptible to agonist-induced short-term desensitization, which distinguishes it from the EP2 subtype. Truncation of the carboxy terminal domain abolishes short-term desensitization[16] and internalization[17]. The EP4 receptor also undergoes down-regulation following long-term exposure to agonist (>12 h), however, down-regulation is also seen with the EP2 receptor.

DP receptor

The DP receptor is the only class of receptor identified to date that mediates the actions of PGD2[15]. Selective DP agonists include 9-deoxy-D^9-PGD2. Several DP receptor antagonists are available including BWA868C. PGD2 exerts its effect by causing an increase in intracellular cyclic AMP. The DP receptor cDNA codes for a protein of 359 amino acids and the recombinant receptor couples to stimulation of adenylyl cyclase. The evidence for DP receptors on platelets is based on the fact that PGD2 inhibits platelet aggregation.

Adenosine receptors

There are four distinct adenosine receptors named A1, A2A, A2B and A3. Platelets contain A2A receptors[18]. Adenosine acts via an A2A receptor to stimulate adenylyl cyclase in platelets and inhibits platelet activation. Mice lacking A2A receptors exhibit slight increases in platelet aggregation[19]. ADP released from platelets can be catabolized to adenosine by ectonucleotidases. This may provide an important mechanism for limiting inappropriate platelet aggregation in an intact blood vessel.

Receptors coupled to inhibition of adenylyl cyclase

In all cases, receptors coupled to inhibition of adenylyl cyclase have no measurable effect on basal adenylyl cyclase activity. In order to demonstrate a Gi or Gz-dependent decrease in adenylyl cyclase, it must be activated with one of the agonists discussed above or by direct stimulation with forskolin.

EP3 receptor

PGE2 acts through the EP3 receptor to inhibit adenylyl cyclase[15]. Selective EP3 agonists include sulprostone, and enprostil. The human EP3 receptor occurs as at least eight isoforms that are the product of alternative splicing of a single gene[20,21]. The isoforms are identical over the first 369 amino acids but differ in carboxyl-terminal region, which varies in length from 6–65 amino acids following the seventh transmembrane helix. While all of the EP3 isoforms couple to inhibition of adenylyl cyclase, they differ in properties including susceptibility to desensitization and degree of constitutive activity. Some of the isoforms also couple to Ca^{2+} mobilization. The EP3 receptor has been functionally demonstrated on platelets by the action of the selective agonist, sulprostone in inhibiting adenylyl cyclase[22]. Co-localization of the EP3 receptor on platelets with the IP, EP2 or EP4, and DP receptors, all of which couple to stimulation of adenylyl cyclase, has led to the description of a novel mechanism of desensitization involving distinct stimulatory and inhibitory receptors[23]. Since prostaglandins are local mediators their concentration might be expected to vary as the autocoid is released and diffuses away from its site of action so that the role of the inhibitory receptor may be to provide homeostatic control of cyclic AMP level, buffering against rapid variations in agonist concentration. Recent studies on EP3 knockout mice indicate that loss of the EP3 receptor in a model of venous inflammation protects against formation of intravascular clots[24].

ADP receptor

The recently cloned P2Y12 receptor has been shown to be coupled to inhibition of adenylyl cyclase[25,26]. While it has been known for a relatively long time that ADP can inhibit activated adenylyl cyclase, the separation of the action of ADP effects on platelets into a Gi-mediated response via a $P2T_{AC}$ receptor (P2Y12) and a Gq-coupled P2Y1 was made only recently[27].

Cambridge Books Online © Cambridge University Press, 2009

Epinephrine receptor

Platelets contain α2a adrenergic receptors that had long been thought to be coupled to the inhibition of adenylyl cyclase via Gi[28]. Recently, it was shown in mice lacking Gz, that epinephrine induced-inhibition of cyclic AMP formation was lacking and aggregation was impaired[29]. The Gz deficient mice were also more resistant to fatal thromboembolism than normal mice.

Thromboxane and thrombin receptors

The question of whether thromboxane A2 receptors in platelets are coupled to adenylyl cyclase inhibition has been controversial. Ushhibucki et al.[30] reconstituted platelet thromboxane A2 into lipid vesicles along with either Gq or Gi2. They found that a thromboxane analogue stimulated GTPγS binding by Gq and Gi2. Hirata et al.[31] showed by RT–PCR that platelets contain cDNA for both thromboxane A2 receptor isozymes. TPα has been shown to couple to activation of adenylyl cyclase and TPβ has been shown to couple to inhibition of cyclase. However, Paul et al.[32] have indicated that the thromboxane mimetic, U46619, does not inhibit adenylyl cyclase directly. Effects of U46619 on cyclic AMP levels can be attributed to secreted autocoids such as ADP or epinephrine. Faruqi et al.[33] have reported that PAR1 but not PAR4 can inhibit platelet adenylyl cyclase in cultured cells. Giesberts et al.[34] showed that the PAR1 specific agonist TRAP can inhibit prostacyclin elevated cyclic AMP. This result has been disputed by Kim et al.[35], who failed to find TRAP-induced adenylyl cyclase inhibition when agents that prevented ADP dependent feedback were included. Thrombin as opposed to the peptides, however, may be able to inhibit adenylyl cyclase

Adenylyl cyclase

There are nine isoforms of adenylyl cyclase that have been cloned and characterized from mammalian sources. The exact isoform of adenylyl cyclase in platelets has not been proven directly. However, several indirect lines of evidence indicate that AC 7 is the major isoform of platelets[36,37]. AC 7 was first found in megakaryocytic HEL cells, which are considered to be a model for platelets. In addition, the level of platelet adenylyl cyclase activity has been proposed to be an indicator of alcoholism[38] and HEK293 cells transfected with AC 7 show greater sensitivity to ethanol than cells expressing other adenylyl cyclase isoforms[39]. AC 7, like most other isoforms, is activated by Gαs and inhibited by Gαi. A brief exposure of cells to ethanol causes an increase in both basal and receptor mediated cyclic AMP production. This effect is probably not direct since ethanol does not activate adenylyl cyclase in membranes. Both ethanol and phorbol dibutyrate (PDBu) treatment enhanced PGE1-stimulated AC activity in HEL cells, platelets and HEK 293 cells transfected with AC 7[40]. Inhibitors of protein kinase C (PKC) blocked the stimulatory effects of both ethanol and PDBu. In cells transfected with AC 7, treatment with either ethanol or PDBu increased the phosphorylation of AC 7. AC 7 was found to be an in vitro substrate of protein kinase C and the use of an isoform specific inhibitor implicated PKC delta in the regulation of AC 7. This study is in contrast to earlier studies of Williams et al.[41], who showed that activation of protein C inhibits the ability of PGE1 to activate platelet adenylyl cyclase. Bushfield et al.[42] also found that phorbol ester treatment of platelets inhibited PGD2 stimulated cyclase, but failed to detect an effect of protein kinase C activation on PGE1-activated adenylyl cyclase.

Guanylyl cyclase

Guanylyl cyclase is found as two major forms, a membrane bound form, which is mainly activated by atrial natriuretic factor (ANF) or intestinal peptide, and soluble guanylyl cyclase that is activated by nitric oxide[43]. The primary form of guanylyl cyclase found in platelets is the soluble form[44]. Soluble guanylyl cyclase is a heterodimer that requires both subunits for activity[43]. Soluble guanylyl cyclase contains a prosthetic heme that is the site for interaction with nitric oxide. Several pharmacologic agents (nitrovasodilators) such as sodium nitroprusside activate guanylyl cyclase by released nitric oxide.

Protein kinases as downstream effectors of cyclic nucleotides

Cyclic AMP dependent kinase

The major, if not only, effect of elevated cyclic AMP levels in platelets is to activate cyclic AMP-dependent protein kinase, also known as protein kinase A (PKA). PKA is a tetramer formed of two catalytic subunits and two regulatory subunits. The regulatory subunits suppress the activity of the catalytic subunits. When the regulatory subunits bind cyclic AMP, they release the catalytic subunits allowing the expression of catalytic activity. Thus, the R-subunits of protein kinase may be detected by its ability to bind

Cambridge Books Online © Cambridge University Press, 2009

cyclic AMP. Using a photoaffinity label, the presence of the regulatory subunits in the particulate fraction in the soluble fraction of platelets has been demonstrated[45]. Using a combination of several techniques, Salama and Haslam[45] have provided convincing evidence for the existence of membrane-bound PKA and soluble PKA in platelets.

Cyclic GMP dependent kinase

Cyclic GMP can alter several effectors but it is probable that the major target in platelets is cyclic GMP-dependent kinase or PKG. PKG is a homodimer. Each subunit contains a catalytic domain and a regulatory domain. When the regulatory domain binds cyclic GMP a conformational change occurs that unmasks the catalytic site. There are two major isozymes of PKG, type I and type II. Platelets contain PKG I[46]. Platelet PKG I but not endothelial or smooth muscle PKG I is essential to prevent intravascular adhesion and aggregation of platelets after ischemia. The defect in platelet PKG I is not compensated by the cyclic AMP/cyclic AMP kinase pathway supporting the essential role of PKG I in prevention of ischemia-induced platelet adhesion and aggregation[47].

Cyclic nucleotide phosphodiesterases

The presence of several different PDE isozymes was initially shown by classical enzyme purification techniques[48]. The members of these PDE families were later cloned yielding six distinct PDE isozymes labeled PDE1-PDE6. More recently, five additional nucleotide sequences encoding additional PDE families (PDE7-PDE11) have been identified[49,50]. Current nomenclature uses arabic numbers to designate 1 of the 11 families with a letter indicating individual genes within a family, and further numerals indicating splice variants[51,52]. A summary of PDE families and their distribution in tissues is reported in Table 20.2. Table 20.3 shows the inhibitors of each PDE isoform.

Table 20.2. Phosphodiesterase (PDE) families and tissue expression

Isoforms	Tissue expression
PDE1	Cardiac muscle, vascular smooth muscle, brain
PDE2	Platelets, cardiac muscle, endothelial cells
PDE3	Platelets, vascular smooth muscle
PDE4	Cardiac muscle, vascular smooth muscle, brain, inflammatory and immune cells
PDE5	Platelets, vascular smooth muscle
PDE6	Retinal rods and cones
PDE7	T-lymphocytes, B-lymphocytes, skeletal muscle, cardiac muscle
PDE8	Testis, eye, liver, kidney, skeletal muscle, embryo, ovary, brain.
PDE9	Small intestinal smooth muscle, liver, kidney, lung, brain, testis, skeletal muscle, hearth, thymus.
PDE10	Testis and brain
PDE11	Skeletal muscle, prostate, kidney, liver, pituitary salivary glands and testis

Table 20.3. Drugs inhibiting PDE isoforms

Isoforms	Drugs
PDE1	Vinpocetine
PDE2	EHNA
PDE3A	Cilostazol, Milrinone, Vesnarinone, and Lixazinone
PDE4	Rolipram, Etazolate, Zardaverine
PDE5	Sidenafil, E4021, DMPPO, Zaprinast, Dypiridamole
PDE6	Sidenafil, Zaprinast, Dypiridamole
PDE8	Dypiridamole
PDE9	Zaprinast
Non-selective	Methylxanthine, theophylline

The major PDE's found in platelets

Hidaka and Asano[48] were the first to resolve the PDE activity of platelets into three distinct peaks. The first enzyme eluted prefers cyclic GMP as substrate with a Km of about 1 mM and is selectively inhibited by PDE5 inhibitors. The second peak of activity hydrolyses both cyclic AMP and cyclic GMP almost equally well[53]. The cyclic AMP activity of enzyme is stimulated by cyclic GMP and selectively inhibited by a PDE2 inhibitor. A third peak of phosphodiesterase activity hydrolyses cyclic AMP much more rapidly than cyclic GMP, has a high-affinity for both cyclic AMP and cyclic GMP and is selectively inhibited by PDE3 inhibitors. The effects of isozyme-selective inhibitors on total PDE in lysed platelets also indicate that these PDE isozymes account for at least 90% of platelet PDE activity. Thus, the best evidence indicates that, in platelets, cyclic AMP is hydrolysed by PDE3 and PDE2 and cyclic GMP is hydrolysed by PDE5 and PDE2.

Cambridge Books Online © Cambridge University Press, 2009

Phosphodiesterase 2 (PDE2) family

PDE2 isozymes were initially named cyclic GMP-stimulated PDE2 because cyclic GMP enhances PDE2 catalyzed cyclic AMP hydrolysis. These enzymes hydrolyse both cyclic AMP and cyclic GMP with similar $Vmax_s$ and cooperative kinetics. They are homodimers of 105 kDa containing two allosteric cyclic GMP binding sites per subunit. One PDE2 gene with three amino-terminal splice variants (PDE2A, PDE2A2 and PDE2A3) has been identified. The only known inhibitor is erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) which can also inhibit adenosine deaminase.

The human platelet PDE2 was purified by affinity chromatography on cyclic GMP-Sepharose. The resulting enzyme had 105 kDa subunits and hydrolysed cyclic AMP and cyclic GMP at similar rates and half-maximal activities were obtained with approximately 50 μM cyclic AMP and 35 μM cGMP[54]. Hydrolysis of both cyclic AMP and cyclic GMP was positively cooperative. In addition, 5–10 μM cyclic GMP stimulated the activity of the purified enzyme up to tenfold. The particular PDE2 isozyme present in platelets has not been identified.

Phosphodiesterase 3 (PDE3)

Members of the PDE3 family have been referred to as cyclic GMP-inhibited cyclic AMP phosphodiesterases, reflecting relatively high affinity for both cyclic AMP and cyclic GMP but a much lower efficacy of hydrolysis for cyclic GMP, which would mean that cyclic GMP may competitively inhibit cyclic AMP hydrolysis by PDE3[55]. There is evidence that this mechanism could occur in platelets in which agents that activate guanylyl cyclase potentiate the effects of activators of adenylyl cyclase[56]. Two genes that encode PDE3 isozymes have been identified. Both cDNAs encode 124 kDa proteins with N-terminal hydrophobic amino acid sequences. PDE3B is membrane-bound and PDE3A is usually a soluble enzyme that is found in platelets[57].

Because of its presence in cardiovascular tissue (platelets, as well as cardiac and vascular smooth muscle) there has been much interest in developing specific inhibitors of PDE3A. Specific PDE3A inhibitors include cilostazol, milrinone, vesnarinone, and lixazinone.

It has been shown that PDE3A can be phosphorylated in intact platelets when cyclic AMP levels are raised. The cyclic AMP phosphodiesterase activity of phosphorylated PDE3A isolated from the platelets shows enhanced activity[58]. The regulation of PDE3A by in vitro phosphorylation through the catalytic subunit of PKA has also been shown to cause an increase in activity[58].

Phosphodiesterase 5 (PDE5)

PDE5s are homodimers with 100 kDa subunits. They are cyclic GMP-specific phosphodiesterases ($K_m \approx 5$ μM) displaying very little cyclic AMP hydrolysis activity. In addition to the catalytic domains, there are two high-affinity non-catalytic cyclic GMP-binding sites homologous to those found in the PDE2 family. One PDE5 gene has been found and two splice variants have been cloned. Three PDE5 selective drugs are available: E4021, DMPPO, and sildenafil. The bovine platelet enzyme has been purified by a combination of chromatography, affinity chromatography and density-gradient centrifugation[59]. The platelet PDE5 has a molecular weight 190 000 and is composed of two subunits (MW 95 000). This enzyme can be phosphorylated by both cyclic AMP- and cyclic GMP-dependent protein kinases, suggesting a possible mechanism of regulation. Platelet PDE5 shows the same relative sensitivity to selective inhibitors as PDE5 from other cells. PDE5 inhibitors alone have little effect on platelet responses to aggregating agents. However, they do potentiate the effects of agents that activate guanylyl cyclase.

Regulation of cyclic AMP levels by phosphodiesterases

With the discovery of type 3 phosphodiesterase and the fact that cyclic GMP is a competitive inhibitor of PDE3, it became important to know whether cyclic GMP-dependent processes had a direct effect on platelet function or whether all cyclic GMP's effects could be explained by elevation of cyclic AMP. Early studies indicated a synergistic interaction between sodium nitroprusside and prostacyclin in inhibiting platelet aggregation[9]. Experiments in which the effect of guanylyl cyclase activators on cyclic AMP levels was measured directly were first done using rabbit platelets. Incubation of the platelets with sodium nitroprusside caused dose-dependent inhibition of platelet function associated with large increases in cyclic GMP and up to 3.0-fold increases in cyclic AMP. However, addition of sodium nitroprusside with a concentration of PGE1 that had little effect alone caused much larger increases in cyclic AMP and greatly enhanced the inhibition of platelet aggregation. Inhibition of adenylyl cyclase with 2′,5′-dideoxyadenosine (DDA) diminished the increases in cyclic AMP caused by nitroprusside in both the presence and absence of PGE1 but reduced the inhibition of platelet function caused by the nitrovasodilators only in the presence of PGE1. These results suggest that, although cyclic GMP may mediate the inhibition of rabbit platelet function

Cambridge Books Online © Cambridge University Press, 2009

by high concentrations of nitrovasodilators added alone, the synergistic interaction of lower concentrations with PGE1 depends on an enhanced accumulation of cyclic AMP. Later, it was shown that in human platelets as well, nitrovasodilators synergize with activators of adenylyl cyclase to inhibit platelet function[60]. However, enhancement of cyclic levels by nitroprusside added alone is limited to 50% in human platelets as opposed to 200% in rabbit platelets.

While it was immediately recognized that PDE3 is an important regulator of cyclic AMP levels, the importance of PDE2 could be questioned since ENHA has no direct effect on cyclic AMP levels[53]. This may be due to the relatively high Km of PDE2 for cyclic AMP. EHNA can cause small increases in the presence of high concentrations of prostacyclin. EHNA potentiated nitroprusside-induced cyclic AMP formation and inhibition of platelet aggregation. Based on these data Haslam et al.[53] proposed 'a PDE inhibitor that acts on both PDE3 and PDE2 could be a particularly potent antithrombotic'.

The effect of the adenylyl cyclase and guanylyl cyclase systems on platelet function

Cyclic AMP

Level of cyclic AMP and inhibition of platelet activation

Inhibition of platelet activation begins when cyclic AMP starts to increase and is proportional to the level of cyclic AMP at the time of response stimulation; the degree of inhibition does not appear to depend on the mechanism by which cyclic AMP is increased[61,62]. This strongly indicates that it is cyclic AMP itself that mediates the inhibition of platelet activation. Small changes in cyclic AMP can have profound effects on platelet activation. For example, about a two- to three-fold rise in cyclic AMP above basal can inhibit ADP-induced aggregation and a 20% increase can inhibit by about 50%[62]. However, one should be cautioned that all the measured basal cyclic AMP may not be available to activate protein kinase A.

Protein phosphorylation induced by elevated cyclic AMP

Incubation of $^{32}PO_4$-labelled platelets with agonists that elevate cyclic AMP causes prominent labelling of two proteins with molecular weights of 24 kDa (P24) and 50 kDa (P50) within the time period required for development of inhibition of platelet responses[63]. P24 was found to be enriched in particulate fractions containing storage granules, mitochondria, and microsomes while P50 was confined to the soluble fraction[64]. P24 was proposed to have a function in regulation of cytosolic Ca^{2+} concentration but has been shown to be the β-subunit of glycoprotein 1b[65]. Platelet responses to agonists are still inhibited by elevating cyclic AMP in Bernard–Soulier platelets, which lack GP1b.

In the early studies of Haslam's group[63], other proteins with molecular weights of 22, 36, 49, and 82 kDa are also phosphorylated. However, these phosphorylations occur too slowly to be of importance for inhibition of platelet response. The 22 kDa protein has been identified as rap1b, a member of the ras super family of small GTP-binding proteins[66]. However, rap1b evidently plays no role in inhibition of platelet function[66] because when it was completely converted into its phosphorylated form in iloprost (a stable form of prostacyclin) pretreated platelets and the iloprost was removed, the platelets that contained phosphorylated rap1b were found to respond when challenged with a wide variety of agonists.

The 49 kDa protein is likely to be the 46/50 kDa vasodilator-stimulated phosphoprotein (VASP). VASP is associated with microfilaments and concentrated at sites of focal adhesion plaques. VASP is phosphorylated by both PKG and PKA at three sites[67]. Both kinases phosphorylate the 'serine 1 site' rapidly and the threonine 1 site 'slowly' in vitro. However, PKG phosphorylates the 'serine 2 site' more rapidly than PKA. In intact platelets both serine sites are phosphorylated in response to either cyclic GMP or cyclic AMP elevation. In mice in which the VASP gene was deleted, cyclic AMP- and cGMP-mediated inhibition of platelet aggregation required significantly higher concentrations of either cyclic nucleotide[68]. Cyclic-nucleotide-mediated inhibition of increases in cytosolic Ca^{2+} and granule secretion were not altered by the absence of VASP. These authors suggest that cyclic nucleotide-dependent phosphorylation of VASP may involve regulation of integrin function.

Myosin light chain kinase (MLCK) has been shown to be a substrate for cyclic AMP-dependent protein kinase[69]. Phosphorylation of MLCK interferes with the ability of calmodulin to bind to MLCK and thus causes an overall inhibition of MLCK.

Actin-binding protein (ABP; 250 kDa), thought to stabilize actin filaments, is also phosphorylated in platelets. ABP phosphorylation was induced by prostaglandin E1 (PGE1) in intact platelets and a platelet kinase preparation phosphorylates ABP in the presence of cAMP[70]. In the PGE1– treated platelets, ABP was proteolysed at a slower rate than in control platelets indicating the cAMP-

Cambridge Books Online © Cambridge University Press, 2009

dependent phosphorylation of ABP may protect ABP from proteolysis by calpain.

The effect of elevated cyclic AMP on platelet signal transduction pathways

When platelets are activated, there is an increased phosphorylation of a 20 kDa protein (myosin light chain) and 47 kDa protein (pleckstrin). Inhibition of platelet responses by substances that elevate cyclic AMP is accompanied by a substantial decrease in the phosphorylation state of both the P20 and P47 proteins. For myosin light chain, the decrease may be explained by inactivation of Ca^{2+}-calmodulin-dependent myosin light chain kinase by phosphorylation by the catalytic subunit of cyclic AMP-dependent protein kinase. However, the physiological significance has been questioned for smooth muscle relaxation[71]. Increase in cyclic AMP also causes decrease in the agonist-stimulated cytosolic concentration of Ca^{2+} which also may contribute to the inactivation of the myosin light chain kinase. For P47, the decrease is also most likely due to inhibition of its kinase, protein kinase C, through lowering of the concentration of its activators Ca^{2+} and diacylglycerol. Elevated cyclic AMP levels in activated platelets reverses the formation of the cytoskeleton[72].

The level of cytosolic Ca^{2+} increases rapidly when platelets are stimulated. This increase is inhibited by prior elevation of the cyclic AMP level of the platelets[73]. Elevating cyclic AMP after platelet activation reverses the activation process which is accompanied by a decrease in the cytosolic Ca^{2+} level[73]. These observations suggest that the cytosolic Ca^{2+} level in platelets can be controlled by cyclic AMP.

Platelet activation by agonists causes hydrolysis of phosphatidylinositol and polyphosphoinositols with a corresponding formation of inositol phosphates and diacylglycerol, the latter being rapidly phosphorylated to phosphatidic acid. Elevation of cyclic AMP has been shown to inhibit agonist-induced accumulation of phosphatidic acid and diacylglycerol[74]. Rink et al.[75] showed that the slow activation of platelets by direct activation of protein kinase C by phorbol ester or exogenous DG is not inhibited by elevation of cyclic AMP.

Stimulation of cyclic AMP formation has no effect on the conversion of added arachidonic acid to prostaglandins and other metabolites, while the agonist-induced liberation of the fatty acid from endogenous phospholipids is inhibited[76]. These results suggest that, while neither cyclooxgenase nor thromboxane synthetase is inhibited by elevated cyclic AMP, phospholipase A2 is inhibited.

Cyclic AMP-stimulated uptake of Ca^{2+} by platelet microsomal particles

Microsomal preparations from platelets have been shown to accumulate Ca^{2+} in an ATP-dependent manner. It has been shown that this uptake is stimulated by addition of cyclic AMP and PKA. During cyclic AMP-stimulated uptake, a protein with a molecular weight of about 22 KDa is phosphorylated and when the membrane vesicles were prepared from platelets with elevated cyclic AMP, the microsomal vesicles contained phosphorylated P24 and had greater Ca^{2+} uptake than membranes from control platelets[77]. This group has suggested that the calcium pump in the membrane vesicles may be regulated by cyclic AMP-dependent phosphorylation of a P22–24 protein and that this pump may be important in regulating Ca^{2+} levels in the platelet cytoplasm and controlled by external agonists via cyclic AMP. While two proteins in the range have been identified, neither can regulate the Ca^{2+} pump since one is rap1b and the other is part of glycoprotein 1b. Adunyah and Dean[78] showed that antibodies raised against phospholamban (the regulator of the sacroplasmic reticular Ca^{2+} pump in the heart) did not crossreact with the platelet 22 kDa protein. In addition, when the platelet protein was boiled in sodium dodecylsulfate, it did not dissociate into lower molecular weight subunits characteristic of cardiac phospholamban. Recently, Dean et al.[79] showed that elevated cyclic AMP resulted in phosphorylation of the platelet membrane Ca^{2+} pump on a tyrosine residue. This phosphorylation was found to inhibit the activity of this pump.

Cyclic AMP-dependent regulation of the IP3 receptor

The IP3 receptor is a receptor operated Ca^{2+} channel that is opened by an increase in cytosolic inositol 1,4,5-trisphosphate (IP_3). Contrary to earlier reports, O'Rourke et al.[80] found that PKA-dependent phosphorylation of the IP3 receptor had no effect on its ability to release stored Ca^{2+}. Quinton et al.[81] found that IP_3 receptor was initially dephosphorylated, its rate of Ca^{2+} release was not inhibited by cyclic AMP-dependent phosphorylation. However, the rate of Ca^{2+} release from IP_3 receptor that had been phosphorylated by endogenous kinase showed a twofold greater rate of Ca^{2+} release and phosphorylation by PKA inhibited this rate by 50% back to the dephosphorylated rate. In studies on living megakaryocytes, Tertyshnikova and Fein[82] concluded that the 'principal mechanism of cyclic AMP-dependent inhibition of Ca^{2+} mobilization in

Cambridge Books Online © Cambridge University Press, 2009

megakaryocytes appears to be by inhibition of IP3-induced Ca^{2+} release'. Wojcikiewicz and Luo[83] found that all three types of IP_3 receptors could be phosphorylated by PKA. However, they found that in a variety of permeabilized cells types there was a slight increase in sensitivity to IP_3 if the IP_3 receptor was phosphorylated irrespective of the isoforms of IP_3 receptor present.

The effect of cyclic AMP on collagen-stimulated platelet activation

Most of the conclusions about the effect of increases in cyclic AMP and cyclic GMP on collagen-stimulated platelet activation were drawn from experiments using agonists whose receptors couple through guanine nucleotide binding proteins (G proteins) to their effector enzyme, usually phospholipase C. The exact mechanism by which collagen activates platelets was unclear at this time and experiments were done with low concentrations of collagen which relied on the products of secretion such as ADP and products of arachidonic acid metabolism such as thromboxane A2 to activate the majority of the platelets. Researchers studying adhesion of platelets to collagen routinely used an agent that elevated cyclic AMP in the incubation buffer. In many cases, elevation of cyclic AMP cause enhanced adhesion of platelets to collagen[84]. Surprisingly, Smith et al. found that several agents that elevated cyclic AMP failed to inhibit collagen-induced Ca^{2+} mobilization but enhanced the level of Ca^{2+} mobilization commensurate with the increase in adhesion. In addition, and in contrast to activation by thrombin, neither collagen-induced secretion nor phosphatidic acid formation was decreased by elevation of cyclic AMP. In addition, the collagen-induced tyrosine phosphorylation of platelet proteins was not inhibited by elevation of platelet cyclic AMP levels[85]. The significance of these results became clearer when it was proposed that collagen signal transduction occurs through a tyrosine kinase-dependent activation of phospholipase $C\gamma2$[86,87]. This mechanism for collagen-induced signalling is now well established and significantly different from the mechanism for G protein-coupled receptors. Figure 20.2 shows the effect of elevation of either cyclic AMP or cyclic GMP on collagen-induced Ca^{2+} mobilization. While ADP-induced Ca^{2+} mobilization is substantially inhibited by elevated cyclic nucleotides, collagen mediated Ca^{2+} signalling is almost unaffected either in the absence or presence of the excellular calcium chelator EGTA. In the case of carbacyclin-treated platelets, some enhancement of Ca^{2+} mobilization is seen due to enhanced adhesion[84].

Consistent with the effect of collagen, Riondino et al.[88] found that it is possible to bypass cyclic AMP's inhibitory effect on fibrinogen binding site exposure induced by the thromboxane A2 synthetic analogue U46619, the snake venom toxin convulxin, or by the direct PKC activator OAG, by concomitantly activating a Gi-coupled receptor by means of epinephrine or by inducing cytosolic calcium influx by means of ionomycin.

Level of cyclic GMP and inhibition of platelet activation

The effects of elevated cyclic GMP are difficult to distinguish from those of cyclic AMP. Again, a small increase in cyclic GMP appears to be sufficient for inhibition of platelet activation by agonists. A major problem is the fact that increases in cyclic GMP result in increases in cyclic AMP via inhibition of PDE3. In addition, there is evidence for cross-talk between the two cyclic nucleotide systems at the level of protein kinases[53]. For example, cyclic AMP can activate PKG I.

Protein phosphorylation induced by elevated cyclic GMP

There are no major differences in the proteins phosphorylated in platelets when cyclic GMP is elevated from those seen with the elevation of cyclic AMP[62]. As mentioned above, it has been shown that VASP is a substrate for both PKA and PKG[67]. In addition, the IP3 receptor appears to be phosphorylated by cyclic GMP-dependent mechanisms[82].

Protein kinase G I deficient mice

One of the best pieces of evidence implicating the cyclic GMP pathway is from a mouse model in which the mice are PKG I-deficient[47]. The authors studied platelet–endothelial cell and platelet–platelet interactions in an ischemia/reperfusion model. Platelet PKG I but not endothelial or smooth muscle PKG I was found to be essential to prevent intravascular adhesion and aggregation of platelets after ischemia. The authors conclude: 'the defect in platelet PKG I is not compensated by the cAMP/cAMP kinase pathway supporting the essential role of PKG I in prevention of ischemia-induced platelet adhesion and aggregation.'

Cambridge Books Online © Cambridge University Press, 2009

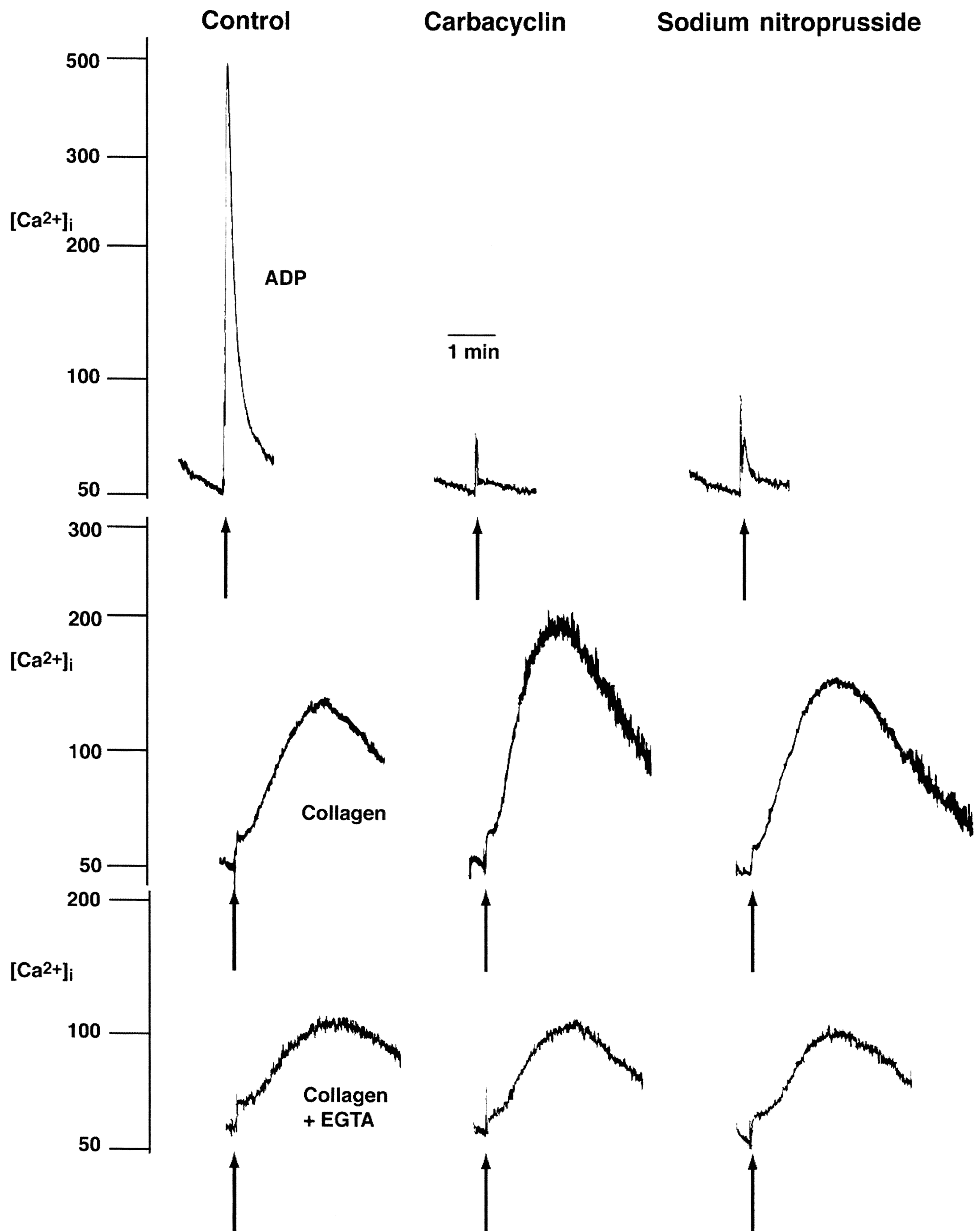


Fig. 20.2. The effect of elevated cyclic nucleotides on collagen-induced Ca^{2+} mobilization. Platelets were aspirinated and loaded with Fura-2. Collagen samples were treated with creatine phosphate (20 mM), creatine phosphokinase (50 U/ml), and 1 mM SC57101 (an aggregation inhibitor). ADP samples contained 1 μM SC57101. Collagen samples were stimulated with 50 μg/ml of Horm collagen. ADP samples were stimulated with 10 μM ADP. The EGTA samples contained 0.5 mM EGTA. Cyclic AMP was elevated with 100 nM Carbacyclin an analogue of prostacyclin. Cyclic GMP levels were elevated with 100 μM sodium nitroprusside.

Cambridge Books Online © Cambridge University Press, 2009

Conclusions

After one third of a century the exact mechanism by which cyclic nucleotides inhibit platelet function remains unknown. Of the several possible mechanisms, none can fully explain the difference between the effects of elevated cyclic nucleotides on G protein-coupled events and collagen-induced platelet activation. It seems likely that this difference points to either G proteins themselves or one of the various isoforms of phospholipase C as targets of cyclic nucleotide regulation. There has been one report that PKG II can regulate Gαq through both phosphorylation of Gαq and by binding to Gαq[89]. Since G protein-coupled agonists activate phospholipase C of the β isoform, the effects of cyclic nucleotides could be explained if β but not γ2 isoforms of phospholipase C were inhibited by cyclic nucleotide-dependent phosphorylation. It has been shown that PKA directly phosphorylates serine residues of the PLC-beta 2 in vivo and in vitro[90]. Cyclic AMP-dependent protein kinase specifically inhibits G$\beta\gamma$-activated PLC-beta 2 activity. Recently, it has been shown that both phospholipase C-β2 and phospholipase C-β3 can be phosphorylated by both PKA and PKG[91,92] by expressing phospholipase C-β3 in COS cells this group showed that phosphorylation of a specific serine inhibited Gαq-stimulated PLCβ3 activity. Whether this mechanism accounts for cyclic nucleotide-dependent inhibition of platelet activation remains to be seen. However, inhibition of PLCβ would account for the difference seen between platelet activation by collagen and by other agonists.

References

1 Marcus AJ, Zucker MB. *The Physiology of Blood Platelets.* New York: Grune and Stratton; 1965.

2 Ardlie NG, Glew G, Schultz BG, Schwartz CJ. Inhibition and reversal of platelet aggregation by methyl xanthines. *Thromb Diath Haemorrh* 1967; 18: 670–673.

3 Wolfe SM, Shulman NR. Adenyl cyclase activity in human platelets. *Biochem Biophys Res Commun* 1969; 35: 265–272.

4 Salzman EW, Neri LL. Cyclic 3′,5′-adenosine monophosphate in human blood platelets. *Nature* 1969; 224: 609–610.

5 Abdulla YH. Beta-adrenergic receptors in human platelets. *J Atheroscler Res* 1969; 9: 171–177.

6 Marquis NR, Vigdahl RL, Tavormina PA. Platelet aggregation. I. Regulation by cyclic AMP and prostaglandin E1. *Biochem Biophys Res Commun* 1969; 36: 965–972.

7 Salzman EW. Cyclic AMP and platelet function. *N Engl J Med* 1972; 286: 358–363.

8 Haslam RJ, Davidson MM, Desjardins JV. Inhibition of adenylate cyclase by adenosine analogues in preparations of broken and intact human platelets. Evidence for the unidirectional control of platelet function by cyclic AMP. *Biochem J* 1978; 176: 83–95.

9 Levin RI, Weksler BB, Jaffe EA. The interaction of sodium nitroprusside with human endothelial cells and platelets: nitroprusside and prostacyclin synergistically inhibit platelet function. *Circulation* 1982; 66: 1299–1307.

10 Mellion BT, Ignarro LJ, Ohlstein EH, Pontecorvo EG, Hyman AL, Kadowitz PJ. Evidence for the inhibitory role of guanosine 3′, 5′-monophosphate in ADP-induced human platelet aggregation in the presence of nitric oxide and related vasodilators. *Blood* 1981; 57: 946–955.

11 Glass DB, Gerrard JM, Townsend D, Carr DW, White JG, Goldberg ND. The involvement of prostaglandin endoperoxide formation in the elevation of cyclic GMP levels during platelet aggregation. *J Cyclic Nucleotide Res* 1977; 3: 37–44.

12 Davies T, Davidson MM, McClenaghan MD, Say A, Haslam RJ. Factors affecting platelet cyclic GMP levels during aggregation induced by collagen and by arachidonic acid. *Thromb Res* 1976; 9: 387–405.

13 Smyth EM, Nestor PV, FitzGerald GA. Agonist-dependent phosphorylation of an epitope-tagged human prostacyclin receptor. *J Biol Chem* 1996; 271: 33698–33704.

14 Jaschonek K, Faul C, Schmidt H, Renn W. Desensitization of platelets to iloprost. Loss of specific binding sites and heterologous desensitization of adenylate cyclase. *Eur J Pharmacol* 1988; 147: 187–196.

15 Coleman RA, Smith WL, Narumiya S. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol Rev* 1994; 46: 205–229.

16 Bastepe M, Ashby B. The long cytoplasmic carboxyl terminus of the prostaglandin E2 receptor EP4 subtype is essential for agonist-induced desensitization. *Mol Pharmacol* 1997; 51: 343–349.

17 Desai S, April H, Nwaneshiudu C, Ashby B. Comparison of agonist-induced internalization of the human EP2 and EP4 prostaglandin receptors: role of the carboxyl terminus in EP4 receptor sequestration. *Mol Pharmacol* 2000; 58: 1279–1286.

18 Huttemann E, Ukena D, Lenschow V, Schwabe U. Ra adenosine receptors in human platelets. Characterization by 5′-N-ethylcarboxamido[3H]adenosine binding in relation to adenylate cyclase activity. *Naunyn Schmiedebergs Arch Pharmacol* 1984; 325: 226–233.

19 Ledent C, Vaugeois JM, Schiffmann SN et al. Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. *Nature* 1997; 388: 674–678.

20 Kotani M, Tanaka I, Ogawa Y et al. Molecular cloning and expression of multiple isoforms of human prostaglandin E receptor EP3 subtype generated by alternative messenger RNA splicing: multiple second messenger systems and tissue-specific distributions. *Mol Pharmacol* 1995; 48: 869–879.

21 Schmid A, Thierauch KH, Schleuning WD, Dinter H. Splice variants of the human EP3 receptor for prostaglandin E2. *Eur J Biochem* 1995; 228: 23–30.

Cambridge Books Online © Cambridge University Press, 2009

22 Mao GF, Jin JG, Bastepe M, Ortiz-Vega S, Ashby B. Prostaglandin E2 both stimulates and inhibits adenyl cyclase on platelets: comparison of effects on cloned EP4 and EP3 prostaglandin receptor subtypes. *Prostaglandins* 1996; 52: 175–185.
23 Ashby B. Novel mechanism of heterologous desensitization of adenylate cyclase: prostaglandins bind with different affinities to both stimulatory and inhibitory receptors on platelets. *Mol Pharmacol* 1990; 38: 46–53.
24 Fabre JE, Nguyen M, Athirakul K et al. Activation of the murine EP3 receptor for PGE2 inhibits cAMP production and promotes platelet aggregation. *J Clin Invest* 2001; 107: 603–610.
25 Foster CJ, Prosser DM, Agans JM et al. Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. *J Clin Invest* 2001; 107: 1591–1598.
26 Hollopeter G, Jantzen HM, Vincent D et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* 2001; 409: 202–207.
27 Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. *Proc Natl Acad Sci USA* 1998; 95: 8070–8074.
28 Kobilka BK, Matsui H, Kobilka TS et al. Cloning, sequencing, and expression of the gene coding for the human platelet alpha 2-adrenergic receptor. *Science* 1987; 238: 650–656.
29 Yang J, Wu J, Kowalska MA et al. Loss of signaling through the G protein, Gz, results in abnormal platelet activation and altered responses to psychoactive drugs. *Proc Natl Acad Sci USA* 2000; 97: 9984–9989.
30 Ushikubi F, Nakamura K, Narumiya S. Functional reconstitution of platelet thromboxane A2 receptors with Gq and Gi2 in phospholipid vesicles. *Mol Pharmacol* 1994; 46: 808–816.
31 Hirata T, Ushikubi F, Kakizuka A, Okuma M, Narumiya S. Two thromboxane A2 receptor isoforms in human platelets. Opposite coupling to adenylyl cyclase with different sensitivity to Arg60 to Leu mutation. *J Clin Invest* 1996; 97: 949–956.
32 Paul BZ, Jin J, Kunapuli SP. Molecular mechanism of thromboxane A(2)-induced platelet aggregation. Essential role for p2t(ac) and alpha(2a) receptors. *J Biol Chem* 1999; 274: 29108–29114.
33 Faruqi TR, Weiss EJ, Shapiro MJ, Huang W, Coughlin SR. Structure-function analysis of protease-activated receptor 4 tethered ligand peptides. Determinants of specificity and utility in assays of receptor function. *J Biol Chem* 2000; 275: 19728–19734.
34 Giesberts AN, van Willigen G, Lapetina EG, Akkerman JW. Regulation of platelet glycoprotein IIb/IIIa (integrin alpha IIB beta 3) function via the thrombin receptor. *Biochem J* 1995; 309: 613–620.
35 Kim S, Quinton TM, Jin J, Cattaneo M, Kunapuli SP. Evidence for diverse signal transduction pathways in thrombin receptor activating peptide (SFLLRN) and other agonist-induced fibrinogen receptors in platelets. *Blood* 2000; 96: 1037a.
36 Hellevuo K, Welborn R, Menninger JA, Tabakoff B. Human adenylyl cyclase type 7 contains polymorphic repeats in the 3′ untranslated region: investigations of association with alcoholism. *Am J Med Genet* 1997; 74: 95–98.
37 Hellevuo K, Berry R, Sikela JM, Tabakoff B. Localization of the gene for a novel human adenylyl cyclase (ADCY7) to chromosome 16. *Hum Genet* 1995; 95: 197–200.
38 Menninger JA, Baron AE, Conigrave KM et al. Platelet adenylyl cyclase activity as a trait marker of alcohol dependence. WHO/ISBRA Collaborative Study Investigators. International Society for Biomedical Research on Alcoholism. *Alcohol Clin Exp Res* 2000; 24: 810–821.
39 Yoshimura M, Tabakoff B. Ethanol's actions on cAMP-mediated signaling in cells transfected with type VII adenylyl cyclase. *Alcohol Clin Exp Res* 1999; 23: 1457–1461.
40 Rabbani M, Nelson EJ, Hoffman PL, Tabakoff B. Role of protein kinase C in ethanol-induced activation of adenylyl cyclase. *Alcohol Clin Exp Res* 1999; 23: 77–86.
41 Williams KA, Murphy W, Haslam RJ. Effects of activation of protein kinase C on the agonist-induced stimulation and inhibition of cyclic AMP formation in intact human platelets. *Biochem J* 1987; 243: 667–678.
42 Bushfield M, Hopple SL, Gibson IF, Murdoch FA, MacIntyre DE. Effects of protein kinase C activation on human platelet cyclic AMP metabolism. *FEBS Lett* 1987; 222: 299–304.
43 Lucas KA, Pitari GM, Kazerounian S et al. Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol Rev* 2000; 52: 375–414.
44 Buechler WA, Ivanova K, Wolfram G, Drummer C, Heim JM, Gerzer R. Soluble guanylyl cyclase and platelet function. *Ann NY Acad Sci* 1994; 714: 151–157.
45 Salama SE, Haslam RJ. Characterization of the protein kinase activities of human platelet supernatant and particulate fractions. *Biochem J* 1984; 218: 285–294.
46 Schmidt HH, Lohmann SM, Walter U. The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. *Biochim Biophys Acta* 1993; 1178: 153–175.
47 Massberg S, Sausbier M, Klatt P et al. Increased adhesion and aggregation of platelets lacking cyclic guanosine 3′,5′-monophosphate kinase I. *J Exp Med* 1999; 189: 1255–1264.
48 Hidaka H, Asano T. Human blood platelet 3′: 5′-cyclic nucleotide phosphodiesterase. Isolation of low-Km and high-Km phosphodiesterase. *Biochim Biophys Acta* 1976; 429: 485–497.
49 Soderling SH, Bayuga SJ, Beavo JA. Isolation and characterization of a dual-substrate phosphodiesterase gene family: PDE10A. *Proc Natl Acad Sci USA* 1999; 96: 7071–7076.
50 Fawcett L, Baxendale R, Stacey P et al. Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A. *Proc Natl Acad Sci USA* 2000; 97: 3702–3707.
51 Beavo JA. Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol Rev* 1995; 75: 725–748.
52 Perry MJ, Higgs GA. Chemotherapeutic potential of phosphodiesterase inhibitors. *Curr Opin Chem Biol* 1998; 2: 472–481.
53 Haslam RJ, Dickinson NT, Jang EK. Cyclic nucleotides and phosphodiesterases in platelets. *Thromb Haemost* 1999; 82: 412–423.
54 Grant PG, Mannarino AF, Colman RW. Purification and charac-

Cambridge Books Online © Cambridge University Press, 2009

terization of a cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from the cytosol of human platelets. *Thromb Res* 1990; 59: 105–119.
55 Grant PG, Colman RW. Purification and characterization of a human platelet cyclic nucleotide phosphodiesterase. *Biochemistry* 1984; 23: 1801–1807.
56 Maurice DH, Haslam RJ. Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: inhibition of cyclic AMP breakdown by cyclic GMP. *Mol Pharmacol* 1990; 37: 671–681.
57 Degerman E, Belfrage P, Manganiello VC. Structure, localization, and regulation of cGMP-inhibited phosphodiesterase (PDE3). *J Biol Chem* 1997; 272: 6823–6826.
58 Macphee CH, Reifsnyder DH, Moore TA, Lerea KM, Beavo JA. Phosphorylation results in activation of a cAMP phosphodiesterase in human platelets. *J Biol Chem* 1988; 263: 10353–10358.
59 Robichon A. A new cGMP phosphodiesterase isolated from bovine platelets is substrate for cAMP- and cGMP-dependent protein kinases: evidence for a key role in the process of platelet activation. *J Cell Biochem* 1991; 47: 147–157.
60 Dickinson NT, Jang EK, Haslam RJ. Activation of cGMP-stimulated phosphodiesterase by nitroprusside limits cAMP accumulation in human platelets: effects on platelet aggregation. *Biochem J* 1997; 323: 371–377.
61 Ashby B, Kowalska MA, Wernick E, Rigmaiden M, Daniel JL, Smith JB. Differences in the mode of action of 1-oleoyl-2-acetyl-glycerol and phorbol ester in platelet activation. *J Cyclic Nucleotide Protein Phosphor Res* 1985; 10: 473–483.
62 Haslam RJ, Davidson MM, Davies T, Lynham JA, McClenaghan MD. Regulation of blood platelet function by cyclic nucleotides. *Adv Cyclic Nucleotide Res* 1978; 9: 533–552.
63 Haslam RJ, Lynham JA, Fox JE. Effects of collagen, ionophore A23187 and prostaglandin E1 on the phosphorylation of specific proteins in blood platelets. *Biochem J* 1979; 178: 397–406.
64 Fox JE, Say AK, Haslam RJ. Subcellular distribution of the different platelet proteins phosphorylated on exposure of intact platelets to ionophore A23187 or to prostaglandin E1. Possible role of a membrane phosphopolypeptide in the regulation of calcium-ion transport. *Biochem J* 1979; 184: 651–661.
65 Fox JE, Berndt MC. Cyclic AMP-dependent phosphorylation of glycoprotein Ib inhibits collagen-induced polymerization of actin in platelets. *J Biol Chem* 1989; 264: 9520–9526.
66 Siess W, Grunberg B. Phosphorylation of rap1B by protein kinase A is not involved in platelet inhibition by cyclic AMP. *Cell Signal* 1993; 5: 209–214.
67 Butt E, Abel K, Krieger M et al. cAMP- and cGMP-dependent protein kinase phosphorylation sites of the focal adhesion vasodilator-stimulated phosphoprotein (VASP) in vitro and in intact human platelets. *J Biol Chem* 1994; 269: 14509–14517.
68 Aszodi A, Pfeifer A, Ahmad M et al. The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function. *EMBO J* 1999; 18: 37–48.
69 Hathaway DR, Eaton CR, Adelstein RS. Regulation of human platelet myosin light chain kinase by the catalytic subunit of cyclic AMP-dependent protein kinase. *Nature* 1981; 291: 252–256.
70 Zhang Z, Lawrence J, Stracher A. Phosphorylation of platelet actin binding protein protects against proteolysis by calcium dependent sulfhydryl protease. *Biochem Biophys Res Commun* 1988; 151: 355–360.
71 Kamm KE, Stull JT. The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annu Rev Pharmacol Toxicol* 1985; 25: 593–620.
72 Feinstein MB, Egan JJ, Opas EE. Reversal of thrombin-induced myosin phosphorylation and the assembly of cytoskeletal structures in platelets by the adenylate cyclase stimulants prostaglandin D2 and forskolin. *J Biol Chem* 1983; 258: 1260–1267.
73 Feinstein MB, Egan JJ, Sha'afi RI, White J. The cytoplasmic concentration of free calcium in platelets is controlled by stimulators of cyclic AMP production (PGD2, PGE1, forskolin). *Biochem Biophys Res Commun* 1983; 113: 598–604.
74 Rittenhouse SE, Allen CL. Synergistic activation by collagen and 15-hydroxy-9 alpha,11 alpha- peroxidoprosta-5,13-dienoic acid (PGH2) of phosphatidylinositol metabolism and arachidonic acid release in human platelets. *J Clin Invest* 1982; 70: 1216–1224.
75 Rink TJ, Sanchez A. Effects of prostaglandin I2 and forskolin on the secretion from platelets evoked at basal concentrations of cytoplasmic free calcium by thrombin, collagen, phorbol ester and exogenous diacylglycerol. *Biochem J* 1984; 222: 833–836.
76 Gerrard JM, Peller JD, Krick TP, White JG. Cyclic AMP and platelet prostaglandin synthesis. *Prostaglandins* 1977; 14: 39–50.
77 Kaser-Glanzmann R, Gerber E, Luscher EF. Regulation of the intracellular calcium level in human blood platelets: cyclic adenosine 3′,5′-monophosphate dependent phosphorylation of a 22,000 dalton component in isolated Ca^{2+}-accumulating vesicles. *Biochim Biophys Acta* 1979; 558: 344–347.
78 Adunyah SE, Jones LR, Dean WL. Structural and functional comparison of a 22 kDa protein from internal human platelet membranes with cardiac phospholamban. *Biochim Biophys Acta* 1988; 941: 63–70.
79 Dean WL, Chen D, Brandt PC, Vanaman TC. Regulation of platelet plasma membrane Ca^{2+}-ATPase by cAMP-dependent and tyrosine phosphorylation. *J Biol Chem* 1997; 272: 15113–15119.
80 O'Rourke F, Zavoico GB, Feinstein MB. Release of Ca^{2+} by inositol 1,4,5-trisphosphate in platelet membrane vesicles is not dependent on cyclic AMP-dependent protein kinase. *Biochem J* 1989; 257: 715–721.
81 Quinton TM, Brown KD, Dean WL. Inositol 1,4,5-trisphosphate-mediated Ca^{2+} release from platelet internal membranes is regulated by differential phosphorylation. *Biochemistry* 1996; 35: 6865–6871.
82 Tertyshnikova S, Fein A. Inhibition of inositol 1,4,5-trisphosphate-induced Ca^{2+} release by cAMP- dependent protein kinase in a living cell. *Proc Natl Acad Sci USA* 1998; 95: 1613–1617.
83 Wojcikiewicz RJ, Luo SG. Phosphorylation of inositol 1,4,5-trisphosphate receptors by cAMP- dependent protein kinase.

Cambridge Books Online © Cambridge University Press, 2009

Type I, II, and III receptors are differentially susceptible to phosphorylation and are phosphorylated in intact cells. *J Biol Chem* 1998; 273: 5670–5677.

84 Smith JB, Dangelmaier C, Selak MA, Ashby B, Daniel J. Cyclic AMP does not inhibit collagen-induced platelet signal transduction. *Biochem J* 1992; 283: 889–892.

85 Smith JB, Dangelmaier C, Daniel JL. Elevation of cAMP in human platelets inhibits thrombin- but not collagen-induced tyrosine phosphorylation. *Biochem Biophys Res Commun* 1993; 191: 695–700.

86 Blake RA, Schieven GL, Watson SP. Collagen stimulates tyrosine phosphorylation of phospholipase C-gamma 2 but not phospholipase C-gamma 1 in human platelets. *FEBS Lett* 1994; 353: 212–216.

87 Daniel JL, Dangelmaier C, Smith JB. Evidence for a role for tyrosine phosphorylation of phospholipase C gamma 2 in collagen-induced platelet cytosolic calcium mobilization. *Biochem J* 1994; 302: 617–622.

88 Riondino S, Gazzaniga PP, Pulcinelli FM. Evidence for platelet alphaIIb beta3 activation despite elevated cytosolic cAMP. *Thromb Haemost* 2001; 85: 320–325.

89 Sallese M, Mariggio S, D'Urbano E, Iacovelli L, De Blasi A. Selective regulation of Gq signaling by G protein-coupled receptor kinase 2: direct interaction of kinase N terminus with activated galphaq. *Mol Pharmacol* 2000; 57: 826–831.

90 Liu M, Simon MI. Regulation by cAMP-dependent protein kinase of a G-protein-mediated phospholipase C. *Nature* 1996; 382: 83–87.

91 Xia C, Bao Z, Yue C, Sanborn BM, Liu M. Phosphorylation and regulation of G-protein-activated phospholipase C- beta 3 by cGMP-dependent protein kinases. *J Biol Chem* 2001; 276: 19770–19777.

92 Yue C, Dodge KL, Weber G, Sanborn BM. Phosphorylation of serine 1105 by protein kinase A inhibits phospholipase Cbeta3 stimulation by Galphaq. *J Biol Chem* 1998; 273: 18023–18027.

Cambridge Books Online © Cambridge University Press, 2009

21

Platelet adhesion

Philip G. de Groot and Jan J. Sixma

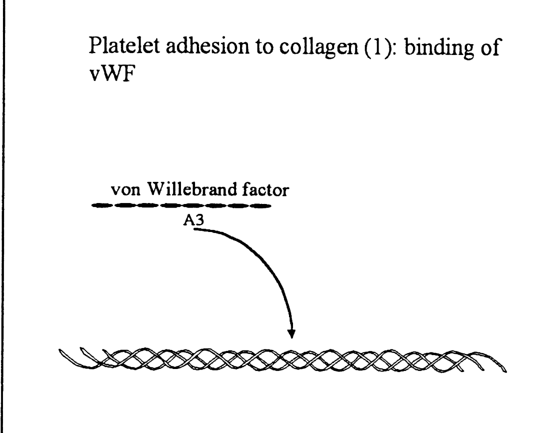
Department of Haematology, University Medical Center, Utrecht, The Netherlands

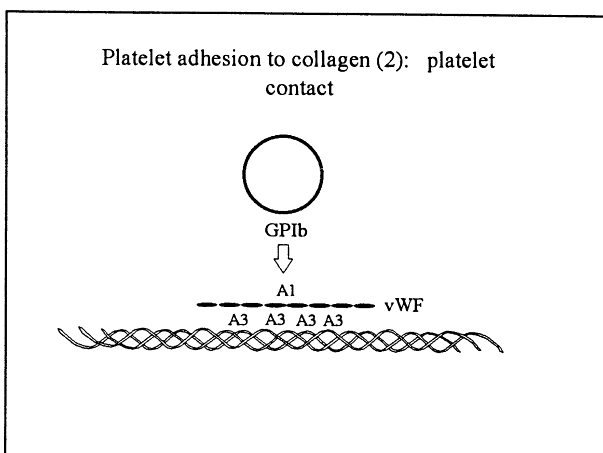
Platelets contribute to the maintenance of the normal circulation of blood through the vasculature by taking care of the integrity of the vessel wall[1]. They continuously 'examine' the inner lining of the vessel wall for leakage without adhering to the intact endothelial cell lining. They respond immediately, however, when the endothelium is injured and they rapidly adhere to the exposed subendothelial structures[2]. After the adherence of a first layer of platelets a series of complicated reactions are initiated that finally result in the formation of a platelet plug stabilized by an insoluble fibrin network. This so-formed hemostatic plug prevents further loss of blood. Platelets, however, cannot distinguish between a dissected vessel wall, which results in bleeding wounds, and superficial lesions of the endothelium occurring in atherosclerosis. Adhesion of platelets to these superficial lesions may be the onset of the formation of a plug in an intact vessel, a mural thrombus that occludes a blood vessel and prevents the supply of blood to a vital organ, resulting in a serious pathological condition, such as heart attack and stroke.

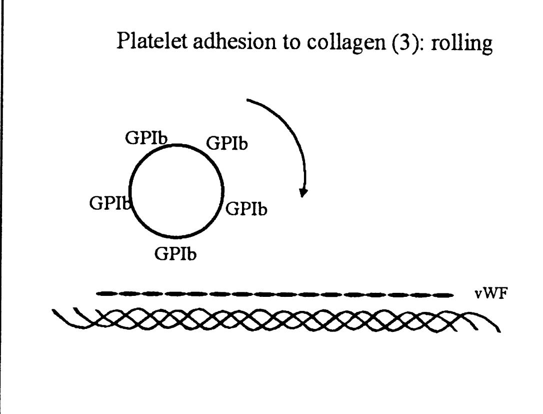
Platelet adhesion is the first step in the hemostatic response and knowledge of the mechanisms of platelet adhesion is an important lead to the development of selective antithrombotic drugs. The adhesive proteins responsible for optimal platelet adhesion are different from adhesive proteins involved in the adhesion of other cells. Platelet adhesion has several unique features. Platelet adhesion occurs in flowing blood, which means that the adhesive process must occur rapidly and the interaction must be firm enough to resist the shear forces of the flowing blood. This has led to the evolution of adhesive receptors and ligands that are unique for platelet adhesion.

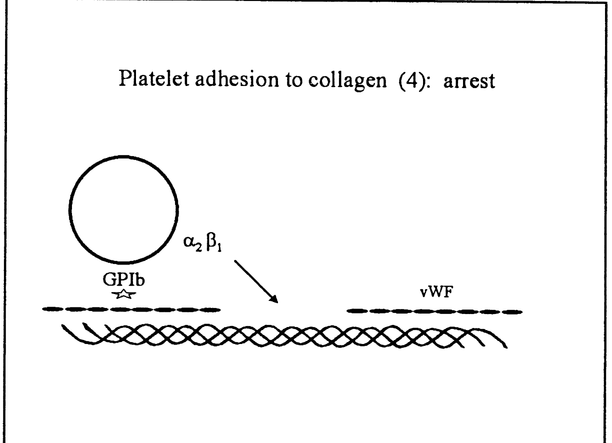
General mechanism of adhesion

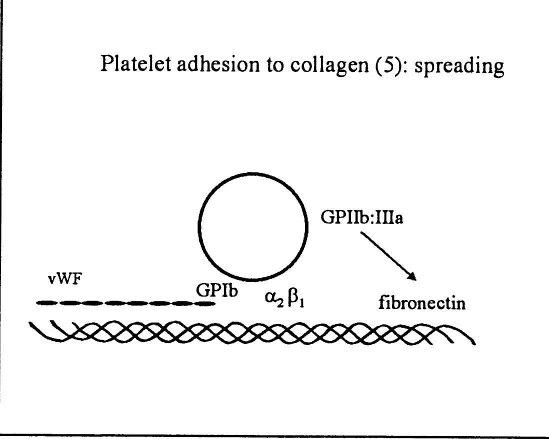
Before the first interaction of a platelet with the injured vessel wall, von Willebrand factor present in plasma will bind to exposed collagens present in the vessel wall[3]. The initial contact of platelets can then take place via the interaction of the platelet receptor complex GPIb/V/IX with the immobilized von Willebrand factor (vWF)[4]. This interaction initiates the tethering of circulating platelets to the vessel wall[5]. Platelets roll over von Willebrand factor in the direction of flow driven by the shear forces (Fig 21.1). A continuous loss of GPIb–vWF interactions at one side of the platelet and the formation of new interactions at the other side of the platelet support the rolling process[6]. The rolling of the platelet will finally end in firm attachment through the participation of other platelet membrane receptors, some of which become available via activation of the rolling platelets. The adhesion of platelets to subendothelium involves, besides the GPIb/V/IX complex, the functioning of at least four other receptors: the collagen receptors $\alpha_2\beta_1$[7] and GPVI[8,9], the fibronectin receptor $\alpha_5\beta_1$[10] and the fibrinogen receptor $\alpha_{IIb}\beta_3$ (GPIIbIIIa)[11]. Spreading of the platelet will follow the firm adhesion and is essential to withstand the shear forces exerted by the flowing blood. GPIIbIIIa mediates the spreading of the platelets[12]. Before the firm adhesion and spreading is established, part of the platelets will detach and return to the circulation[13]. The spread platelet is a new surface for a next platelet to adhere to and is the basis of a platelet aggregate. Historically, platelet adhesion and platelet aggregate formation were considered as two separate stages of the formation of a thrombus. Nowadays, the similarities between adhesion and aggregation are more prominent[14,15]. For both adhesion and aggregation, circulating platelets must attach to an adhesive protein, while shear forces are generated on the platelet by the flowing blood. An adhered and subse-











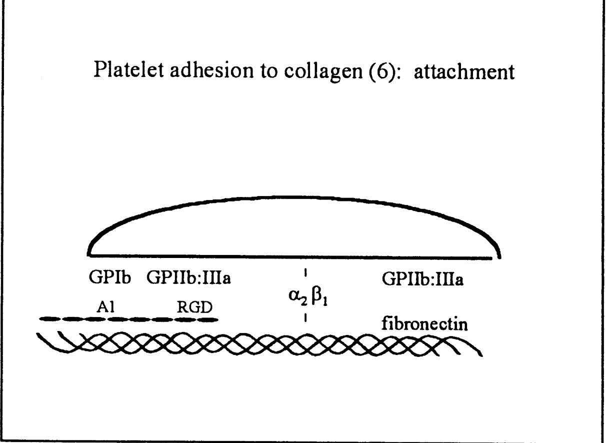


Fig. 21.1. Sequence of events in platelet adhesion.

Cambridge Books Online © Cambridge University Press, 2009

quently spread platelet binds fibrinogen and von Willebrand factor from the circulation to GPIIbIIIa and GPIb, respectively, creating an ideal surface for the next platelet to adhere. Although platelet adhesion is dependent on additional receptors such as $\alpha_2\beta_1$ and aggregation is strongly supported by responses to thrombin and ADP, the fundamental mechanism for both processes, an interaction between a platelet receptor (GPIIbIIIa and GPIb/V/IX) with its substrate (fibrinogen and vWF) in flowing blood is similar[16,17,18]. This is highlighted by the important observation that, both for adhesion and aggregation, the importance of von Willebrand factor increases with increasing shear rate.

Influence of shear stress

Knowledge of the rheological factors that influence the adhesion of platelets was greatly enhanced by the development of perfusion chambers[19]. These chambers enable the study of platelet adhesion under conditions of flow. The relevant fluid dynamic factors to describe the influence of flow on platelet adhesion are shear rate (γ) and shear stress (τ)[20]. The fluid shear stress is defined as the tangential force per unit area exerted in the direction of flow. In case of a liquid obeying Newton's Second Law, the shear stress is proportionally related to the shear rate: $\tau = \eta.\gamma$ in which η is the viscosity of the blood. Shear rates and shear stresses can be calculated for various parts of the vasculature from the known vascular diameter and volume flow rates (Table 21.1). The shear rates are low in large vessels but increase up to 5000 s^{-1} in capillaries. The shear rates in stenosed arteries are even much higher. One should keep in mind that the calculations of shear rate and shear stress are based on a simplified model. All the calculations are based on a steady flow and not on pulsatile flow. The calculations are also related to laminar flow, while branching and abnormal vascular curvature will strongly influence rheology. Also blood is not a Newtonian fluid, the viscosity of blood is not constant but decreases with increasing shear rates. The non-Newtonian flow properties of whole blood are due to the presence of red blood cells as they are the major part of the cellular volume of the blood. Nevertheless the use of shear rates and shear stress enables us to describe the role of blood flow in platelet adhesion.

One of the main factors determining platelet adhesion is the transport of platelets to the vessel wall surface. This process is driven by the shear rate of the blood in combination with the presence of red cells. Platelet adhesion increases with increasing shear rate up to about 2000 s^{-1}. At higher shear rates, platelet adhesion remains more or less unchanged till shear rates of 5000 s^{-1}. The distribution of red cells in flowing blood is not homogeneous. Red cells are concentrated in the middle of the stream, while the smaller platelets are pushed aside towards the vessel surface due to collisions with the red cells. The consequence is that the local concentration of platelets near the vessel wall increases with increasing shear rate. The increased local platelet concentration is one of the main reasons for the increased adhesion found with increasing shear rate. Two rate-limiting mechanisms can be distinguished for platelet adhesion[21]. When the wall shear rate is low (<300 s^{-1}), the adhesion is diffusion controlled. When the wall shear rate is high (>1300s^{-1}), the adhesion is reaction controlled. In the area in-between both shear rates both processes contribute.

Table 21.1. Fluid dynamic parameters

Vessel	Diameter (cm)	Wall shear rate (s^{-1})	Wall shear stress (dyne/cm^2)
Aorta ascendens	2.3–4.5	50–300	2–10
Arteria femoralis	0.5	350	11
Small arteries	0.03	1500	55
Capillaries	0.0006	2000–5000	[a]
Large veins	0.5–10	200	7
Vena cava inferior	2.0	50	2
Stenosed arteries	0.025	40 000	3000

Notes:
The results presented are from references [20, 133, 134, 135, 136] and [137].
[a] Calculations are not meaningful since flow regime is totally different and bulk viscosity is not applicable.

The transport of platelets by the red cells is dependent on the hematocrit, red cell rigidity and red cell size. The rotation of the red cells enhances the diffusion of the platelet to the vessel wall[22]. This effect is dependent on the rigidity of the red cells. Together with the plasma viscosity the red cell rigidity determines the viscosity of the blood[23]. An increase in plasma viscosity will result in an increase in platelet deposition[24]. The increased blood viscosity found in patients with, e.g. diabetes mellitus might explain in part the increased thrombotic tendency that has been found in these patients.

The influence of shear forces on platelet adhesion is depicted in Fig. 21.2. The influence of shear can be described by the factors influencing the concentration of platelets in the boundary layer near the vessel surface (diffusion controlled) and by the affinity of the receptors for their adhesive proteins in combination with the forces exerted on the platelet (reaction controlled). Platelet con-

Cambridge Books Online © Cambridge University Press, 2009

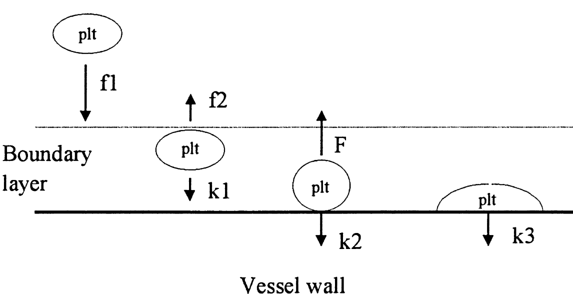


Fig. 21.2. Schematic representation of the hemodynamic and biochemical processes involved in platelet adhesion under flow. Platelet concentration in the boundary layer is increased due to convective diffusion of platelets from the bulk concentration (f1) and Brownian diffusion from the boundary layer (f2), resulting in an equilibrium concentration. Platelets will attach with a certain rate (k1) and will adhere with certain affinity (k2). After spreading of the cells the affinity increases (k3). Shear forces (F) will detach (a fraction of the) platelets resulting in transport back into the bulk concentration.[137].

centration in the boundary layer is determined by convective diffusion from the bulk concentration and Brownian diffusion, resulting in an equilibrium concentration. Platelets will attach to proteins present in the vessel wall with a certain velocity (k_1), adhere with a certain affinity (k_2) and firmly attach with another affinity (k_3). Shear forces will try to detach platelets (F) resulting in a platelet transport from the wall.

Platelet–vessel wall interaction at the molecular level, the adhesive proteins

Various components of the subendothelium, including von Willebrand factor, various types of collagen, fibronectin and fibrinogen, can act as substrate for platelet adhesion[25]. Most studies on platelet adhesion have been performed with isolated proteins and we can now describe and understand the interaction of platelets with single proteins. The individual contributions of these proteins to the interaction of platelets with a much more complex subendothelium remain to be investigated. On the one hand, there may be synergy of multiple substrate receptor interactions whereas on the other hand, mutual interactions between proteins in the subendothelium might specifically shield off certain adhesive domains. It is apparent, however, that vWF is essential and unique in tethering rapidly flowing platelets to an exposed thrombogenic surface.

Von Willebrand factor

vWF is an adhesive protein that circulates in plasma but that is also present in the subendothelium and in α-granules of platelets. It is synthesized by endothelial cells and megakaryocytes[26,27]. The average plasma concentration is 10 µg/ml, the average concentration in the platelet is 280 ng/10^9 platelets[28]. The normal range of plasma vWF, however, is broad and is strongly influenced by the blood group with 25% lower levels in blood group O and high levels in persons with the AB loci[29]. Platelet vWF is independent of ABO blood type. vWF is a multimeric protein that interacts with ligands in the subendothelium and on platelets. It bridges the platelet and the surface that otherwise poorly interact under conditions of shear. The multimeric structure is essential for its biological activity. Low molecular weight forms as found in patients with type 2A von Willebrand disease lack adhesive activity[30]. The multimeric structure results from connection of two C-termini by disulfide bonds followed by disulfide bonds between N-termini, resulting in a long thread with a molecular weight of over 20 million[31]. vWF in the circulation does not bind spontaneously to platelets. When damage of the vascular surface occurs, vWF will bind immediately to the exposed collagens[3]. The interaction with collagen results in 'activation' of vWF, which enables the recognition of vWF by platelet GPIb/V/IX complex. The activation of vWF must be a conformational change, but the mechanism of activation has not been elucidated yet. Recently, the conformational change due to a gain of function mutation in vWF has been elucidated by crystallization of the A1 domain of a I546V mutant[32]. The I546V substitution alters the charge distribution within the proposed GPIb binding site at a significant distance from the mutation. The interaction between vWF and platelet glycoprotein GPIb can also be promoted in vitro by an obsolete antibiotic ristocetin and the snake venom protein botrocetin[33,34] or by mutations in vWF or GPIb[35]. These mutations in vWF and GPIb are also effective in vivo.

Mature vWF-monomer is built up of a number of domains[36] (Fig. 21.3). These domains are thought to be independent structural and functional units of the protein. The domains are arranged in the order D–D3–A1–A2–A3–D4–B1–B2–B3–C1–C2–CK. The A1- and the A3-domains in vWF have been identified as the main binding sites for GPIb and collagen, respectively. Within the C1-domain an RGD sequence is present that is responsible for the interaction of vWF with platelet GPIIb–IIIa[37]. Studying loss- or gain-of-function mutations has identified lysine 599 within the vWF A1-domain as essential for the interaction with GPIb[38]. The interaction of ristocetin and botrocetin with vWF could be dissociated within the A1-domain[38].

Cambridge Books Online © Cambridge University Press, 2009

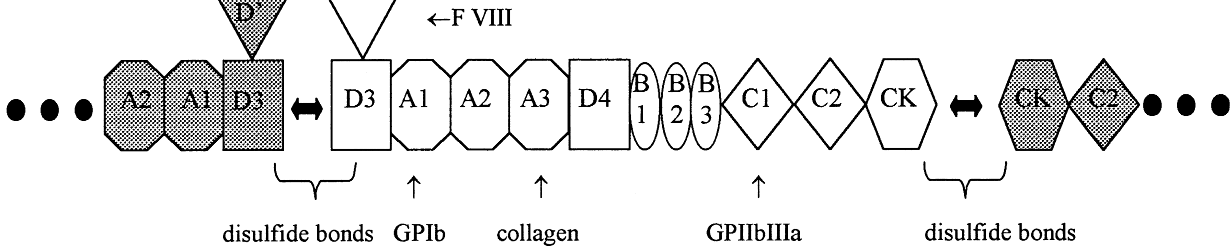


Fig. 21.3. The schematic structure of vWF is shown. The location of binding sites for several ligands are indicated. In the endoplasmic reticulum, von Willebrand monomer is dimerized through disulfide linkage through the cysteine knot (CK domains) at their carboxy termini. In the Golgi large multimers are formed through additional disulfide bonds in the D3-domains.

Ristocetin-induced binding to GPIb was reduced selectively by substitutions in the A1-domain at positions Lys-534, Arg-571, Lys-572, Glu-596, Glu-613, Arg-616, Glu-626, and Lys-642, whereas botrocetin-induced binding to GPIb was decreased selectively by mutations at Arg-636 and Lys-667.

Recently, the crystal structures of both the A1- and the A3-domain have been elucidated[39,40,41]. Both domains are similar in organization and related to A- or I-domains found in integrins. Both domains have a typical α/β fold, consisting of a central hydrophobic parallel β-sheet flanked by amphipathic α-helices on each side. The difference with the homologous A-domains of integrins is that the typical metal ion-dependent adhesion site (MIDAS) motif is incomplete and a metal ion is not present. Consequently, the binding site for GPIb and collagen is not localized in the MIDAS site, where the binding sites are located in integrins.

To localize the collagen-binding site, the crystal structure of a complex of A3 with a Fab fragment of an antibody that inhibits collagen binding was determined. The antibody recognized a non-linear epitope located distant from the top face of the domain, where collagen-binding domains were found in homologous integrin I-domains. The domain consists of sequences 962–966, 981–997 and 1022–1026. With this information as a lead, site-directed mutagenesis was performed that identified His 1023 within the A3-domain as essential for the interaction with collagen type I. Also, mutation of Arg-963 and Arg-1016 reduced collagen binding by 25%[42]. The residues are part of a domain consisting of two β-sheets and one α-helix. His-1023 displays multiple conformations in available A3-crystal structures suggesting that binding of A3 to collagen involves changes in the conformation[40,41].

Collagens

Collagens are distinguishable from other extracellular matrix proteins by their triple helical conformation[43]. All fibrillar collagens have a very similar tertiary protein structure. These collagens are composed of three peptide chains (α-chains). The amino acid sequences of the α-chains show two unique features. There is a glycine at every third residue, generating a repeating $(\text{Gly–X–Y})_n$ pattern and a high proportion of the X and Y residues are proline or hydroxyproline. These features of collagen are essential for the generation and stability of the triple helix. The triple helix conformation consists always of three parallel polypeptide chains that are staggered by one amino acid. The glycine residues are buried in the backbone of the helix while the X and Y are exposed to the solvent[44]. The amino acids at the X and Y position determine the specificity of collagen for certain interactions. The triple helix conformation of collagen is essential for its interaction with cells; a single chain collagen polypeptide has lost its biological activity[45,46]. Apparently, the 3D-conformation of the amino acid sequence is critical. Also platelet adhesion and activation under conditions of flow only occur when the tertiary structure is intact.

At present, 19 different types of collagens have been identified of which collagen types I, III, IV, V, VI, VIII and sometimes XI occur in the vessel wall. These collagens can be broadly divided into reactive and less reactive types for platelets of which collagen types I, III and IV belong to the reactive ones[47]. These three major types of collagen support platelet adhesion also at relatively high shear rates and also cause platelet aggregate formation. These collagens have separate sites for adhesion and aggregation[48]. Platelet adhesion to collagen is strongly affected by the presence of Mg^{2+} and Ca^{2+}[49]. Platelet adhesion to collagen is stimulated by the presence of Mg^{2+} whereas Ca^{2+} has an inhibitory effect. Mg^{2+} mediates its effect via the collagen receptor $\alpha_2\beta_1$. Collagen type VI has been shown to be a binding site for vWF in the subendothelium and, as such, it is involved in platelet adhesion to superficial injuries in vessels[50].

Platelet reactive adhesive domains in collagen are deter-

Cambridge Books Online © Cambridge University Press, 2009

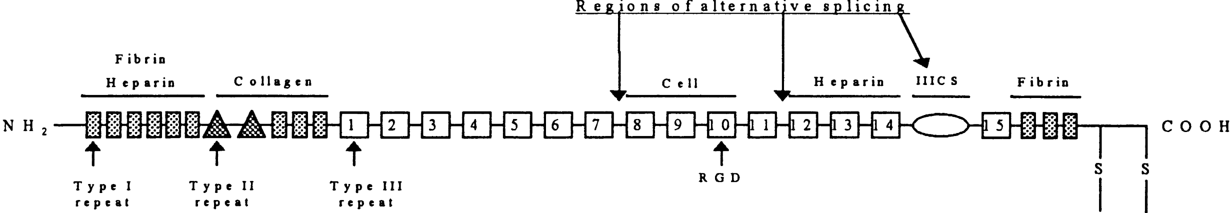


Fig. 21.4. Diagram of the fibronectin subunit.

mined by linear sequences present in one chain. The first sequence described as a recognition site for $\alpha_2\beta_1$, DGEA present within the α1-chain of collagen type I, was suggested to be independent of conformation[51]. However, others were unable to confirm the data and the role of DGEA as an $\alpha_2\beta_1$ recognition site is questionable. Examination of synthetic peptides with the basic structure of collagens, glycine–proline–hydroxyproline $(GPO)_n$, showed that they are very potent activators of platelet aggregation[52]. Polypeptides consisting of GPO-triplets spontaneously form a triple helical conformation and it has been shown that this sequence interacts with the collagen receptor GPVI on platelets. Although the GPO sequence contributes to the overall mechanism of platelet adhesion to collagen, GPVI is the primary collagen receptor on platelets for activation and not for adhesion[53,54]. The primary adhesive receptor on platelets is $\alpha_2\beta_1$[55]. Elegant experiments using overlapping peptides derived from cyanogen bromide fragments of collagen type I, have elucidated an $\alpha_2\beta_1$ recognition site in collagen type I, GFOGER[56]. Triple helical peptides containing this sequence inhibit platelet adhesion not only to collagen type I but also to collagen type III. Recently, the crystal structure has been elucidated of a complex between the I-domain fragment of the α_2-chain of the platelet receptor $\alpha_2\beta_1$ and the synthetic collagen peptide containing the critical GFOGER-sequence that mimics the collagen-binding site[57]. Integrin I-domains contain a divalent cation binding site. In the I-domain of the α_2-chain this metal ion is Mg^{2+}. Three loops on the upper surface of the I-domain provide five amino acid side chains that coordinate the Mg^{2+}. The sixth coordination site of the bound magnesium was provided by the glutamate present in the peptide, completing the coordination sphere of the metal. The interaction between collagen and the I-domain is supported almost exclusively by one of the three α-chains of the triple helix.

An octapeptide sequence KOGEOGPK has been described within the α1-chain of collagen type III (residues 497–504) that was able to support platelet adhesion under conditions of flow[58]. Recently, a new collagen-binding protein, TIIICBP, with a molecular weight of 68 kD, that recognizes this sequence has been isolated[59]. The peptide inhibited platelet adhesion to collagen type III but not to collagen type I. The status of this sequence, however, remains controversial as others never found adhesion to triple helical peptides containing this sequence[60].

Fibronectin

Fibronectin is a ubiquitous glycoprotein composed of two structurally similar but non-identical subunits of approximately 220 kD. The two chains are different due to alternative splicing in the so-called III connecting segment (IIICS)[61]. Two forms of fibronectin exists, a soluble plasma form that is produced by hepatocytes and that consists of a disulfide-bonded dimer[62] and a tissue form of fibronectin that is insoluble and occurs in high molecular weight forms. The tissue fibronectin differs also from plasma fibronectin in an extra type III domain, again due to alternative splicing. Each subunit of fibronectin is composed of three types of homologous repeats, type I, type II and type III. The tenth type III repeat contains the RGD sequence that can interact with GPIIbIIIa or $\alpha_5\beta_1$ on the platelet membrane (Fig. 21.4).

Purified fibronectin supports platelet adhesion and spreading up to shear rates of 1300 s^{-1} [10]. The adhesion is mediated by both $\alpha_5\beta_1$ and GPIIb–IIIa while the spreading is dependent on GPIIb–IIIa. Under conditions of flow, interaction between GPIb and von Willebrand factor is necessary for optimal adhesion[63]. The presence of von Willebrand factor in platelets is sufficient. In a more complex substrate, such as a subendothelium, fibronectin has an additive role. Under these conditions it is not the RGD-site in domain III-10 that is involved, but an, as yet not very well characterized, site in domain III-9[64]. A candidate is the PHSRN sequence in the 9th repeat recognized by the integrin $\alpha_5\beta_1$[65].

Fibrinogen/fibrin

Fibrinogen is not a structural component of the vessel wall, because vessel wall cells do not synthesize it. Nevertheless it is present in vessel wall segments as the result of adsorption from plasma. It is one of the major substrates for

Cambridge Books Online © Cambridge University Press, 2009

platelets. Platelet adhesion to fibrinogen has received much attention, because fibrinogen is the dominant plasma protein to adsorb to biomaterials. As a consequence, the adsorbed fibrinogen provides an ideal surface for platelets to adhere[66]. Also, the adhesion of platelets to a fibrin network is a key event in the maintenance of a hemostatic response. Studies with monoclonal antibodies show that GPIIbIIIa is the major receptor involved in the adhesion to fibrin and fibrinogen at all shear rates[67]. Fibrinogen has three potential reactive domains that are able to interact with GPIIbIIIa. There is a RGDS sequence between residues 572 and 575 and a RGDF sequence between residues 95 and 98 on the α-chain, and AGDV sequence at position 407–411 of the γ-chain. Studies with recombinant fibrinogens have shown that the RGD sequences are not important for platelet adhesion whereas the sequence in the γ-chain is essential[68,69].

Platelet adhesion to fibrin and fibrinogen at high shear rates ($>500\ s^{-1}$) depends not only on the interaction via GPIIbIIIa but also on a secondary interaction between vWF and GPIb[70]. This second interaction is necessary to enable the anchored platelets to withstand the shear forces. The participation of vWF in platelet-fibrin interaction is indirect. vWF must first bind to platelets before it can interact with fibrin. Without platelets no interaction of vWF with fibrin is seen. The underlying molecular mechanism is not clear but may be explained by two effects. First, the local concentration of vWF will be increased as more vWF filaments are held near the surface. This will enhance binding through a mass action effect. Secondly, there is an entropy term that must be considered in any binding reaction. A strong decrease in entropy loss after binding of vWF to platelets may facilitate the interaction of vWF with fibrin.

While non-stimulated platelets express GPIIb–IIIa on the platelet membrane, binding of soluble fibrinogen only takes place after stimulation of platelets with agonists, resulting in a transformation of GPIIb–IIIa into a functional state. However, fibrinogen adsorbed on a surface supports adhesion of non-stimulated platelets via GPIIb–IIIa[71].

Laminin

Laminin is a ubiquitous basement membrane protein[72]. The molecule, as visualized by rotary shadowing, is a cross-shaped molecule with a molecular weight of 850 kDa. It consists of three distinct chains of 400 kD, 215 kD and 205 kD, and it interacts with collagen type IV and heparan sulphate proteoglycans to form the structural part of the basement membrane. Platelet adhesion to laminin is mediated by the integrin $\alpha_6\beta_1$[73]. Adhesion to laminin is strongly dependent on the presence of the divalent cations Mg^{2+} and Ca^{2+}. Antibodies directed against the E8 domain, the terminal half of the long arm, completely inhibit adhesion. The participation of laminin to the platelet reactivity of the more complex subendothelium is unknown.

Platelet-vessel wall interaction at the molecular level: the platelet receptors

Proteins of the integrin family are central molecules in the adhesion process[74]. They mediate cell–cell communications and cell-extracellular matrix interactions. They are heterodimers composed of an α and a β-subunit, both transmembrane glycoproteins. Up to 17 different α-subunits and 8 β-subunits have been identified, leading to a large number of dimers with different biological functions. Platelets contain four integrins involved in platelet–substrate interaction: $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_{IIb}\beta_3$ (= GPIIb–IIIa), the receptors for collagen, fibronectin, laminin and fibrinogen, respectively. Besides integrins also the GPIb–IX–V complex, a platelet specific complex consisting of members belonging to the leucine-rich protein family[4] and GPVI, a member of the immunoglobulin superfamily[75], are essential for optimal platelet adhesion.

Glycoprotein Ib/V/IX

The GPIb–V–IX complex is made up of four distinct gene products[76,77]. The disulfide bonded GPIbα and GPIbβ are non-covalently linked to GPIX and GPV. The four polypeptides GPIbα, GPIBβ, GPIX and GPV are present in a 2:2:2:1 stoichiometry. GPIb–V–IX is constitutively expressed on the platelet surface with about 25 000 copies/platelet. It does not bind spontaneously to vWF, it requires either association of vWF with a surface or, alternatively, exposure of the platelets and vWF to high shear rate in vivo ($>10\,000\ s^{-1}$)[78]. GPIb has also been identified as a thrombin-binding site on platelets[79]. Activation of platelets with thrombin results in a 50% down-regulation of GPIb. The down regulation does not influence platelet adhesion; 10 000 copies of the complex per platelet are enough for optimal adhesion[80]. A number of studies have localized the vWF-binding site to the N-terminal domain of GPIα[81]. The most potent inhibitor is a 45kD N-terminal fragment (His1–Arg293). This suggests that the interaction of the complex with vWF requires conformational dependent epitopes that could not be mimicked by linear peptides. The binding site for vWF within this domain consists of more than one site. One binding site is the sulfated tyrosine sequence Tyr276–Glu282 containing sulfated tyrosines at Tyr-276, Tyr-278 and Tyr-279[82]; the other binding site is located in the leucine-rich repeats 2, 3, and 4 of GPIbα[83].

Congenital abnormalities present in three chains of the complex, GPIbα, GPIbβ and GPIX are associated with the

Cambridge Books Online © Cambridge University Press, 2009

Bernard–Soulier syndrome. Most of the mutations found lead to truncated or defective polypeptides and are not informative for the localization of the binding site. Two mutations have been described leading to in-frame substitutions in the first and sixth leucine-rich repeat resulting in a dysfunctional GPIb complex[84,85]. Whether these mutations disrupt an adhesive site or the conformation of the whole domain is unknown.

GPIIb–IIIa

Glycoprotein IIb–IIIa ($\alpha_{IIb}\beta_3$) is the most abundant platelet receptor[86]. It is a receptor for fibrinogen, fibronectin, vitronectin, von Willebrand factor and thrombospondin. It is not only involved in platelet aggregation but also in platelet adhesion. Both the α-subunit and the β-subunit on GPIIb–IIIa contribute to binding of ligands[87]. It is not known whether the α- and β-subunit ligand binding regions form one extended binding area or are separate binding sites recognizing different epitopes in the ligand. The amino-terminal 334 amino acids of α_{IIb} have been implicated in the ligand binding[88]. Mutations in the sequence 184–193 (GAPGGYYFLG) significantly decrease binding of $\alpha_{IIb}\beta_3$ to soluble fibrinogen without affecting the expression of $\alpha_{IIb}\beta_3$[89]. The sequence between the disulfide bond Cys-146–Cys-167 is also involved in ligand binding. An (Asp-163–Ala) mutation abolishes ligand-binding properties, indicating that this residue might be critical for ligand binding[90]. The Ca^{2+} binding sites in GPIIb are essential for the maintenance of the proper conformation of GPIIb–IIIa but seem not to be involved in ligand binding.

Thrombopoietin (TPO) is a major regulator of platelet formation in vivo[91]. In vitro studies suggest that TPO sensitizes platelets for activation by several agonists[92]. Experiments in our laboratory have shown that the changed affinity and/or avidity of the integrin GPIIb–IIIa results in an inhibition of platelet rolling and a rapid firm adhesion immediately after the first adhesion. The decreased time period for de-adhesion of platelets results in an increased platelet adhesion to fibrinogen and von Willebrand factor[93]. These observations show that the activation stage of platelet receptors determines the final amount of platelet adhesion.

Collagen receptor α2β1

In 1985, a patient with a hemorrhagic disorder and an excessively long bleeding time was described. The patient's platelets were only non-responsive to collagen, they responded normally to other platelet stimuli. Analysis of the platelet membrane glycoproteins showed that the platelets were deficient in glycoprotein Ia which is the α2-chain of the integrin $\alpha_2\beta_1$[7]. Platelet adhesion to collagen and subendothelium was almost absent[94]. Additional experiments with antibodies and purified receptor confirmed that $\alpha_2\beta_1$ is the major adhesion receptor on the platelet for collagen[95]. Platelet $\alpha_2\beta_1$ levels among randomly selected individuals can vary up to tenfold; this correlates with differences in adhesiveness to type-I or type-III collagens[96]. The N-terminal region of the α2-chain contains seven tandem repeats with internal homology[97]. In addition, it contains an inserted segment of about 200 amino acids in between the second and third repeats. This so-called insertion domain or I-domain has homology with A-domains present in vWF and it contains the collagen-binding site[98].

The I-domain of the α2-chain has been crystallized in the absence and presence of GPO-peptides[58]. Comparison of the liganded domain with the non-liganded I domain reveals a change in metal coordination linked to a reorganization of the upper surface that together create a complementary surface for binding collagen. Conformational changes propagate from the upper surface to the opposite pole of the domain, suggesting both a basis for affinity regulation and a pathway for signal transduction.

According to the crystal structure, the aspartic acid-219 of the I domain forms a salt bridge with Arg in the GFOGER sequence and the side chains of the phenylalanine of this sequence interact with glutamine-215, asparagine-154, tyrosine-157 and leucine-286.

Collagen receptor GPVI

Recently, the platelet collagen receptor glycoprotein (GP) VI was cloned[75]. It codes for a protein of 339 amino acids including a putative 23-amino acid signal sequence and a 19-amino acid transmembrane domain between residues 247 and 265. GPVI belongs to the immunoglobulin superfamily, and its sequence is closely related to $F_c\alpha R$ and to the natural killer receptors. Purified GPVI, as well as Fab fragments of polyclonal antibodies made against the receptor, inhibited collagen-induced platelet aggregation. An arginine residue is found in position 3 of the transmembrane portion, which should permit association with $F_c\gamma$ and its immunoreceptor tyrosine-based activation motif via a salt bridge. With 51 amino acids, the cytoplasmic tail is relatively long and shows little homology to the C-terminal part of the other family members. No information is available yet about domains involved in binding to the GPO motif in collagen and no mutations have been described influencing platelet adhesion.

Fibronectin receptor

The fibronectin receptor $\alpha_5\beta_1$ binds simultaneously to a PHSRN sequence in the 9th type III repeat of fibronectin

Cambridge Books Online © Cambridge University Press, 2009

and the RGD-sequence in the 10th type III repeat[65]. The X-ray crystal structure of a fibronectin fragment reveals that both binding sites are located 35 Å apart on the same side of the molecule[99]. The recombinant fragment 229–448 of the α5-subunit contains the minimal domain for fibronectin recognition[100].

Genetics of platelet adhesion

Platelet thrombus formation is a multistep process determined by the platelet receptors GPIb, GPIIb–IIIa, $\alpha_2\beta_1$ and GPVI and their ligands von Willebrand factor, fibrinogen and collagen. The recent developments in genetic technology have resulted in the discovery of polymorphisms coding for qualitative or quantitative variation of these determinants in platelet thrombus formation. The genetic markers proved to be important determinants to study an etiological relationship between these markers and the risk of cardiovascular diseases in large prospective evaluations. A major functional polymorphism in platelet receptors is a 807T polymorphism of $\alpha_2\beta_1$ coding for an increased density of $\alpha2\beta1$ on the platelet membrane[96]. The presence of this 807 T polymorphism correlates with cardiovascular mortality. In separate studies it was shown that the presence of this 807T polymorphism resulted in an increased adhesion to collagen in vitro[101,102]. Other polymorphisms are a 5T polymorphism of GPIb coding for increased expression of GPIb[103] and a PI^{A2} polymorphism of GPIIbIIIa coding for a change in structure of GPIIb–IIIa[104], both possibly related to increased cardiovascular accidents.

There are also polymorphisms found in matrix proteins. An interesting genetic variation is the blood group difference. Blood groups AA, AB and BB are predictive for cardiovascular disease probably due to the increased levels of vWF in blood group AB[105,106]. A G/T polymorphism in a transcription factor Sp1 binding site of collagen type I[107] and a G/A polymorphism in the promoter region of the fibrinogen β-chain, which is associated with elevated fibrinogen levels[108,109] are other interesting genetic markers for an increased risk of platelet thrombus formation.

Platelet adhesion in disease

Thrombocytopenia

Optimal platelet adhesion depends on the platelet number. The normal range of circulating platelet counts $\times 10^9$ is $150–400 \times 10^9$/l. A good inverse correlation was found between a bleeding time and a platelet count of between 10×10^9/l and 100×10^9/l. At platelet numbers above 100×10^9/l no abnormalities were found[110]. Platelet adhesion studies in vitro showed a similar relation between adhesion and platelet number; 100×10^9 platelets/l are sufficient for optimal adhesion. At higher shear rates fewer platelets were necessary for optimal adhesion. Whether the bleeding tendency at lower platelet count is primarily due to impaired adhesion or impaired aggregate formation is not known. Bleeding tendency as seen in May–Hegglin syndrome and Wiskott–Aldrich syndrome are primarily explained by the low platelet count.

von Willebrand's disease (vWD)

This is characterized by absence or abnormality of vWF and it is one of the most common inherited bleeding diseases[111,112]. The prevalence of vWD has been estimated at about 100 cases per million persons. vWD is classified in three major categories[35]. Type 1 refers to a partial, quantitative deficiency of vWF. vWD type 2 characterizes all qualitative abnormalities in vWF function, whereas type 3 refers to an essentially complete deficiency of vWF. Type 2 is further subdivided into four variants based on differences in structure-function relation. The bleeding symptoms are mild to moderate for most patients, although life-threatening bleeding may occur, especially in vWD type 3. In vitro experiments showed an adhesion defect at shear rates over 500 s^{-1} [113]. Although platelets of vWD patients did not show defects in platelet aggregation in the aggregometer, there is a defect in thrombus formation when the experiments were performed in flowing blood[114]. The lack of thrombus formation in flowing vWD blood becomes more prominent at higher shear rates. When platelets adhere to more reactive surfaces, vWF present in the α-granules of platelets also participates in adhesion[115,116]. As mentioned above, type 1 refers to a quantitative disorder with a normal multimeric distribution. vWF in plasma has apparently a normal function and the bleeding tendency should correlate with the residual amount of vWF in plasma. However, variations in the bleeding tendency have been seen, partly explained by the amount of vWF present in the platelets. Type 2A is characterized by the absence of large multimers of vWF. The large multimers are the effective form of vWF and the functional activity of vWF is much lower than could be explained by the amount of the antigen. The lower multimers lack the ability to bind to collagen while the interaction with GPIb is still present[117]. The lack of ability of type 2A vWF to bind to collagen could contribute to the bleeding disorder. As vWF is also involved in platelet aggregate formation, the ristocetin agglutination is impaired; an impaired aggregate formation could also be the cause of bleeding. Type 2B is defined by an increased affinity of the mutant vWF for platelet GPIb. This results in spontaneous binding of large multimers to platelets in vivo, followed by a rapid clearance of

Cambridge Books Online © Cambridge University Press, 2009

the complex. The remaining multimers are of low molecular weight and less active. The combination of a thrombocytopenia, lower vWF levels and the prevalence of inactive low multimers explains the bleeding disorder[118]. The loss of platelet adhesion is reinforced because once vWF is bound to a platelet it is unable to bind to collagen any longer. The circulating vWF–platelet complexes are hemostatically inactive. The mutations found in type 2B are all found in and around the A1-domain[119]. Type 2M is characterized by vWF with a normal multimeric structure associated with a defective platelet function. This variant is caused by mutations that inactivate the binding site of vWF for GPIb. All the mutations that have been characterized so far are located within the A1-domain[120]. Mutations that result in an inactive binding site for collagen should also be included in this variant, however, until now such mutations have never been found. Type 2N is characterized by mutations in the binding site for factor VIII. The bleeding tendency mimics hemophilia A and no abnormalities in platelet adhesion are found. Type 3 includes patients with vWF levels less than 1% of normal in plasma. There is a secondary deficiency of factor VIII and the patients have a combined defect in primary hemostasis and fibrin formation. The pathology of platelet adhesion is not different from type 1 because both variants are due to a quantitative defect.

Pseudo-vWD has a clinical picture comparable to type 2B. The mutation, however, is not present in vWF but in GPIb[121,122]. The final result is the same, an increased binding of vWF to platelets, resulting in thrombocytopenia, low vWF levels and a prevalence of inactive low multimeric forms of vWF in the circulation.

Acquired vWD is a rare bleeding disorder, which mimics most of the clinical and laboratory features of vWD[123]. The occurrence of acquired vWD is in most cases explained by the presence of circulating autoantibodies to vWF. The presence of these antibodies can result in clearance of antibody-vWF complexes resulting in a deficiency of vWF or the antibodies can inhibit specific functions of vWF such as the interaction of vWF with collagen[124].

Other bleeding disorders

Absence or abnormal function of the GPIIbIIIa complex characterizes Glanzmann's thrombasthenia[125,126]. This defect results in complete absence of platelet aggregation, but in only a limited decrease in platelet adhesion at higher shear rates[11,127]. The loss of platelet adhesion is probably caused by lack of platelet spreading, which is observed at all shear rates. The lack of spreading results in less firmly attached platelets that are more easily detached at shear rates over $1600 s^{-1}$.

Bernard–Soulier syndrome is a form of macrothrombocytopenia characterized by absence or abnormality of GPIb–V–IX complex[128]. In general the combination of a large platelet size and low platelet number results in a total platelet mass that is not reduced in comparison with the normal situation. Comparison of platelet adhesion in macrothrombocytopenia with that of normal platelets at similar platelet counts has shown no adhesion defect. The severe bleeding tendency seen in Bernard–Soulier syndrome is due to a combination of low platelet number and an absence in GPIb–vWF interaction.

Storage pool deficiency is characterized by reduction of the dense granules in platelets, leading to a reduced secretion of ADP after activation. Platelet adhesion is not affected, a reduced aggregate formation explains the bleeding tendency in these patients[11].

No information is available as to whether increased adhesion will lead to thrombotic complications. Increased levels of vWF have been shown to be an increased risk for cardiovascular events but a correlation with increased adhesion has never been shown. Patients with Diabetes mellitus and Hypercholesterolemia, diseases associated with atherosclerotic vessel disease, showed no increased platelet adhesion[129] (P. de Graaf et al., unpublished observations).

Conclusion

Platelet adhesion is a complex process in which many adhesive ligands and their receptors participate. In spite of considerable progress during the last twenty years, initiated by the development of perfusion chambers that enabled the study of platelet adhesion under conditions of flow, the mechanisms that regulate platelet adhesion remain to be elucidated. Which pathways are responsible for the activation of, e.g. GPIIb–IIIa when a platelet is rolling over vWF? Is this process reversible? A mouse model, in which both von Willebrand factor and fibrinogen were deficient, still showed adhesion and platelet aggregation, pointing to other components in the vessel wall and/or plasma capable of supporting adhesion[130]. A mouse model lacking thrombospondin-2 showed an unexplained bleeding diathesis[131] suggesting a role for thrombospondin in the regulation of adhesion[132]. The observation that the severe bleeding tendency of patients with Glanzmann's disease can be treated efficiently with factor VIIa points to a more intensive interaction between platelet function and coagulation than was originally assumed. Also induction of fibrinolysis may quickly reduce the affinity of surfaces such as fibrin for platelets[62]. Another

Cambridge Books Online © Cambridge University Press, 2009

not well explored area is the increased reactivity of atherosclerotic lesions[133] that may be the result of inflammatory modulation of the composition of the plaque. Further experiments with genetically modified animals in combination with appropriate experimental flow models will enhance our ability to understand, diagnose and treat bleeding disorders and arterial thrombosis.

References

1 Sixma JJ, Wester J. The hemostatic plug. *Semin Haematol* 1977; 14: 265–299.
2 Sixma JJ, van Zanten H, Huizinga EG et al. Platelet adhesion to collagen: an update. *Thromb Haemost* 1977; 78: 434–438.
3 Sakariassen KS, Bolhuis PA, Sixma JJ. Human blood platelets adhesion to artery subendothelium is mediated by factor VIII-von Willebrand factor bound to the subendothelium. *Nature* 1979; 279: 636–638.
4 Clemetson KJ, Clemetson JM. Platelet GPIb–V–IX complex. Structure, function, physiology and pathology. *Semin Thromb Haemost* 1995; 21: 130–136.
5 Savage B, Saldivar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell* 1996; 84: 289–297.
6 Miura S, Li CQ, Cao Z, Wang H, Wardell MR, Sadler JE. Interaction of von Willebrand factor domain A1 with platelet glycoprotein Ibalpha-(1–289). Slow intrinsic binding kinetics mediate rapid platelet adhesion. *J Biol Chem* 2000; 275: 7539–7546.
7 Nieuwenhuis HK, Akkerman JWN, Houdijk WPM, Sixma JJ. Human blood platelets showing no response to collagen fail to express surface glycoprotein Ia. *Nature* 1985; 318: 470–473.
8 Arai M, Yamamoto N, Moroi M, Akamatsu N, Fukutaka K, Tanoue K. Platelets with 10% of the normal amount of GPVI have an impaired response to collagen that results in a mild bleeding tendency. *Br J Haemotol* 1995; 89: 357–364.
9 Kehrel B, Clementson KJ, Anders O et al. Glycoprotein VI is a major collagen receptor for platelet activation: it recognizes the platelet-activating quaternary structure of collagen, whereas CD36, glycoprotein IIb/IIIa and von Willebrand factor do not. *Blood* 1998; 91: 491–496.
10 Beumer S, IJsseldijk MJW, de Groot PhG, Sixma JJ. Platelet adhesion to fibronectin in flow: dependence on surface concentration and shear rate, role of platelet membrane glycoproteins GPIIb/IIIa and VLA-5, and inhibition by heparin. *Blood* 1994; 84; 3724–3733.
11 Weiss HJ, Turitto VT, Baumgartner HR. Platelet adhesion and thrombus formation on subendothelium in platelets deficient in glycoproteins IIb–IIIa, Ib, and storage granules. *Blood* 1986; 67: 322–330.
12 Weiss HJ, Turitto VT, Baumgartner HR. Further evidence that glycoprotein IIb–IIIa mediates platelet spreading on subendothelium. *Thromb Haemost* 1991; 65: 202–205.
13 Wu YP, de Groot PhG, Sixma JJ. Shear stress-induced detachment of blood platelets adhered to various adhesive surfaces under flow. *Arterioscler Thromb Vasc Biol* 1997; 17: 3202–3207.
14 Kulkarni S, Dopheide SM, Yap CL et al. A revised model of platelet aggregation. *J Clin Invest* 2000; 105: 783–791.
15 Ruggeri ZM. Old concepts and new developments in the study of platelet aggregation. *J Clin Invest* 2000; 105: 699–701.
16 Ruggeri ZM, Dent JA, Saldivar E. Contribution of distinct adhesive interactions to platelet aggregation in flowing blood. *Blood* 1999; 94: 172–178.
17 Wu YP, Vink T, Schiphorst M et al. Platelet thrombus formation on collagen at high shear rates is mediated by von Willebrand factor-GPIb interaction and inhibited by vWF–GPIIb: IIIa interaction. *Arterioscler Thromb Vasc Biol* 2000; 20: 1661–1667.
18 Tsuji S, Sugimoto M, Miyata S, Kuwahara M, Kinoshita S, Yoshioka A. Real time analysis of mural thrombus formation in various platelet aggregation disorders: distinct shear-dependent roles of platelet receptors and adhesive proteins under flow. *Blood* 1999; 94: 968–975.
19 de Groot PhG, Sixma JJ. Platelet adhesion to the subendothelium under flow. In: *Methods in Molecular Biology* vol 96, Dejana E, Corada M ed. *Protocols for adhesion proteins* Totowa, NJ: Humana Press Inc. 1999; 159–170.
20 Goldsmith HL, Turitto VT. Rheological aspects of thrombosis and haemostasis: basic principles and applications. *Thromb Haemost* 1986; 55: 415–435.
21 Turitto VT, Baumgartner HR. Platelet deposition on subendothelium exposed to flowing blood. Mathematical analysis of physical parameters. *Trans Am Soc Artif Organs* 1975; 21: 593–599.
22 Wang NH, Keller KH. Solute transport induced by erythrocyte motion in shear flow. *Fed Proc* 1979; 30: 1591–1596.
23 Van Breugel HHFI, de Groot PG, Heethaar RM, Sixma JJ. Role of plasma viscosity in platelet adhesion. *Blood* 1992; 80: 953–959.
24 Aarts PAMM, Heethaar RM, Sixma JJ. Red blood cell deformability influences platelet–vessel wall interaction in flowing blood. *Blood* 1984; 64: 1228–1233.
25 Sixma JJ, de Groot PG. Platelet adhesion. In: van Hinsbergh ed. *Vascular control of hemostasis.* (*Advances in Vascular Biology*, volume 1) Harwood Academic Publishers; 1996: 147–168.
26 Jaffe EA, Hoyer LW, Nachman RL. Synthesis of von Willebrand factor by cultured human endothelial cells. *Proc Natl Acad Sci USA* 1974; 71: 1906–1909.
27 Nachman R, Levine R, Jaffe EA. Synthesis of factor VIII antigen by cultured guinea pig megakaryocytes. *J Clin Invest* 1977; 60: 914–921.
28 Rohdeghiero F, Castaman G, Ruggeri ZM, Tosetto A. The bleeding time in normal subjects is mainly determined by platelet von Willebrand factor and is independent from blood group. *Thromb Res* 1992; 65: 605–615.

Cambridge Books Online © Cambridge University Press, 2009

29 Sarode R, Goldstein J, Sussman II, Nagel RL, Tsai HM. Role of A and B blood group antigens in the expression of adhesive activity of von Willebrand factor. *Br J Haematol* 2000; 109: 857–64.
30 Ewenstein BM. Von Willebrand's disease. *Annu Rev Med* 1997; 48: 525–545.
31 Verweij CL, Diergaarde PJ, Hart M, Pannekoek H. Full-length von Willebrand factor cDNA encodes a highly repetitive protein considerably larger than the mature vWF subunit. *EMBO J* 1986; 5: 1839–1847.
32 Celikel R, Ruggeri ZM, Varughese KI. Von Willebrand conformation and adhesive function is modulated by an internalized water molecule. *Nature* 2000; 7: 881–884.
33 Read MS, Potter JY, Brinkhous KM. Venom coagglutinin for detection of von Willebrand factor activity in animal plasmas. *J Lab Clin Med* 1983; 101: 74–82.
34 Ruggeri ZM, Zimmerman TS. von Willebrand factor and von Willebrand disease. *Blood* 1987; 70: 895–904.
35 Sadler JE. A revised classification of von Willebrand disease. *Thromb Haemost* 1994; 71: 520–525.
36 Sadler JE. Biochemistry and genetics of von Willebrand factor. *Annu Rev Biochem* 1998; 67: 395–424.
37 Berliner S, Niija K, Roberts JR, Houghten RA, Ruggeri ZM. Generation and characterization of peptide-specific antibodies that inhibit von Willebrand Factor binding to glycoprotein IIb–IIIa without interaction with other adhesive molecules. *J Biol Chem* 1988; 263: 7500–7505.
38 Matsushita T, Meyer D, Sadler JE. Localization of von Willebrand factor-binding sites for platelet glycoprotein Ib and botrocetin by charged-to-alanine scanning mutagenesis. *J Biol Chem* 2000; 275: 11044–11049.
39 Emsley J, Cruz M, Handin R, Liddington R. Crystal structure of the von Willebrand Factor A1 domain and implications for the binding of platelet glycoprotein Ib. *J Biol Chem* 1998; 273: 10396–10401.
40 Huizinga EG, Martijn van der Plas R, Kroon J, Sixma JJ, Gros P. Crystal structure of the A3 domain of human von Willebrand factor: implications for collagen binding. *Structure* 1997; 5: 1147–1156.
41 Bienkowska J, Cruz M, Atiemo A, Handin R, Liddington R. The von Willebrand Factor A3 domain does not contain a metal ion-dependent adhesion site motif. *J Biol Chem* 1997; 272: 25162–25167.
42 Romijn RA, Bouma B, Wuyster W et al. Identification of the collagen-binding site of the von Willebrand factor A3-domain. *J Biol Chem* 200; 276: 9985–9991.
43 Brodsky B, Ramshaw JAM. The collagen triple helix structure. *Matrix Biol* 1977; 15: 545–558.
44 Jones EY, Miller A. Analysis of structural design features of collagen *JMolBiol* 1991; 218: 209–215.
45 Eble JA, Golbik R, Mann K, Kühn K. The $\alpha1\beta1$ integrin recognition site of the basement membrane collagen molecule [α1(IV)2α2(IV)]. *EMBO J* 1993; 12: 4795–4801.
46 Rao GHR, Fields CG, White JG, Fields GB. Promoting of human platelet adhesion and aggregation by a synthetic, triple-helical mini-collagen. *J Biol Chem* 1994; 269: 13899–13904.
47 Saelman EUM, Nieuwenhuis HK, Hese KM et al. Platelet adhesion to collagen types I through VIII under conditions of stasis and flow is mediated by GPIa/IIa ($\alpha2\beta1$-integrin). *Blood* 1993; 83: 1244–1250.
48 Morton LF, Peachey AR, Barnes MJ. Platelet-reactive sites in collagen type I and type III. Evidence for separate adhesion and aggregatory sites. *Biochem J* 1989; 258: 157–163.
49 Van Zanten GH, Saelman EUM, Schut-Hese KM et al. Platelet adhesion to collagen type IV under flow conditions. *Blood* 1996; 88: 3862–3871.
50 Rand JH, Patel ND, Schwartz E, Zhou SL, Potter BJ. 150 kD-von Willebrand factor binding protein extracted from human vascular subendothelium is type VI collagen. *J Clin Invest* 1991; 88: 263–258.
51 Staatz WD, Fok KF, Zutter MM, Rodriguez BA, Santoro SA. Identification of a tetrapeptide recognition sequence for the a2b1 integrin in collagen. *J Biol Chem* 1991; 266: 7362–7367.
52 Morton LF, Hargreaves PG, Farndale RW, Young RD, Barnes MJ. Integrin $\alpha2\beta1$-independent activation of platelets by simple collagen-like peptides: collagen tertiary (triple helical) and quarternary (polymeric) structure are sufficient alone for $\alpha2\beta1$ independent platelet reactivity. *Biochem J* 1995; 306: 337–442.
53 Moroi M, Jung SM, Shinmyozu K, Tomiyama Y, Ordinas A, Diaz-Ricart M. Analysis of platelet adhesion to a collagen-coated surface under flow conditions: the involvement of glycoprotein VI in the platelet adhesion. *Blood* 1996; 88: 2081–2092.
54 Verkleij MW, Morton LF, Knight CG, de Groot PhG, Barnes MJ, Sixma JJ. Simple collagen-like peptides support platelet adhesion under static but not under flow conditions: Interaction via a2β1 and vWF with specific sequences in native collagen is a requirement to resist shear forces. *Blood* 1998; 91: 3808–3816.
55 Kehrel B. Platelet receptors for collagen. *Platelets* 1995; 6: 11–25.
56 Knight CG, Morton LF, Onley DJ et al. Identification in collagen type I of an integrin $\alpha2\beta1$-binding site containing an essential GER sequence. *J Biol Chem* 1998; 273: 33287–33292.
57 Emsley J, Knight CG, Farndale RW, Barnes MJ, Liddington RC. Structural basis of collagen recognition by integrin $\alpha2\beta1$. *Cell* 2000; 100: 47–56.
58 Karniguian A, Legrand YJ, Lefrancier P, Caen JP. Effect of a collagen derived octapeptide on different steps of the platelet/collagen interaction. *Thromb Res* 1983; 32: 593–604.
59 Monnet E, Fauvel-Lafève F. A new platelet receptor specific to type III collagen. *J Biol Chem* 2000; 275: 10912–10917.
60 Verkleij MW, IJsseldijk MJW, Heijnen-Snyder GJ et al. Adhesive domains in the collagen III fragment α1(III) CB4 that support $\alpha2\beta1$-and von Willebrand factor mediated platelet adhesion under flow conditions. *Thromb Haemost* 1999; 82: 1137–1144.
61 Kornblihtt AR, Umezawa K, Vibe-Pedersen K, Baralle FE.

Cambridge Books Online © Cambridge University Press, 2009

Primary sequence of human fibronectin: differential splicing may generate at least 10 polypeptides from a single gene. *EMBO J* 1985; 4: 1755–1761.
62 Hynes RO. *Fibronectins*. New York: Springer-Verlag; 1990.
63 Beumer S, Heijnen HFG, IJsseldijk MJW, Orlando E, de Groot PG, Sixma JJ. Platelet adhesion to fibronectin in flow: the importance of von Willebrand Factor and glycoprotein Ib. *Blood* 1995; 86; 3452–3460.
64 Beumer S, Heijnen-Snyder G, IJsseldijk MJW, de Groot PhG & Sixma JJ. Fibronectin in an extracellular matrix of cultured endothelial cells supports platelet adhesion via its ninth type III repeat. *Arterioscler Thrombos Vasc Biol* 2000; 20: E16–E19.
65 Loftus JC, Liddington RC. New insights into integrin-ligand interaction. *J Clin Invest* 1997; 99: 2302–2306.
66 Chinn JA, Horbett TA, Ratner BD. Baboon fibrinogen adsorption and platelet adhesion to polymeric materials. *Thromb Haemost* 1991; 65: 608–617.
67 Hantgan RR, Hindriks G, Taylor RG, Sixma JJ, de Groot PG. Glycoprotein Ib, von Willebrand factor, and Glycoprotein IIb: IIIa are all involved in platelet adhesion to fibrin in flowing whole blood. *Blood* 1990; 76: 345–353.
68 Hantgan RR, Endenburg SC, Sixma JJ, & de Groot PG. Evidence that fibrin a-chain RGDX sequences are not required for platelet adhesion in flowing whole blood. *Blood* 1995; 86: 1001–1007.
69 Zaidi TN, McIntire LV, Farrell DH, Thiagarajan P. Adhesion of platelets to surface-bound fibrinogen under flow. *Blood* 1996; 88: 2967–2972.
70 Endenburg SC, Hantgan RR, Lindeboom-Blokzijl L et al. On the role of von Willebrand factor in promoting platelet adhesion to fibrin in flowing blood. *Blood* 1995; 86: 4158–4165.
71 Savage B, Ruggeri ZM. Selective recognition of adhesive sites in surface bound fibrinogen by glycoprotein IIb–IIIa on nonactivated platelets. *J Biol Chem* 1991; 266: 11227–11233.
72 Martin GR, Timpl R, Kuhn K. Laminin and other basement membrane components. *Annu Rev Cell* Biol 1987; 3: 57–71.
73 Hindriks G, IJsseldijk MJW, Sonnenberg A, Sixma JJ, de Groot PG. Platelet adhesion to laminin: Role of Ca^{2+} and Mg^{2+} ions, shear rate and platelet membrane glycoproteins. *Blood* 1992; 79: 928–935.
74 Schwartz MA, Schaller MD, Ginsberg MH. Integrins: emerging paradigms of signal transduction. *Annu Rev Cell Dev Biol* 1995; 11: 549–599.
75 Clemetson JM, Polgar J, Magnenat E, Wells TN, Clemetson KJ. The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcalphaR and the natural killer receptors. *J Biol Chem* 1999; 274: 29019–29024.
76 Lopez JA. The platelet glycoprotein GPIb-IX complex. *Blood Coagul Fibrinol* 1994; 5: 97–119.
77 Andrews RK, Shen Y, Gardiner EE, Dong JF, Lopez JA, Berndt MC. The glycoprotein Ib–IX–V complex in platelet adhesion and signaling. *Thromb Haemost* 1999; 82: 357–364.
78 Kroll MH, Hellums JD, McIntyre LV, Schafer AI, Moake JL. Platelets and shear stress. *Blood* 1996; 88: 1525–1541.
79 Marchese P, Murata M, Mazzucato M et al. Identification of three tyrosine residues of glycoprotein Ibα with distinct roles in von Willebrand factor and thrombin binding. *J Biol Chem* 1995; 270: 9571–9578.
80 Van Zanten GH, Heijnen HFG, Wu YP et al. A 50% reduction of platelet surface glycoprotein Ib does not affect platelet adhesion under flow conditions. *Blood* 1997; 91: 2353–2359.
81 Ware J. Molecular analyses of the platelet glycoprotein Ib–IX–V receptor. *Thromb Haemost* 1998; 79: 466–478.
82 Dong JF, Li CQ, Lopez JA. Tyrosine sulfation of the glycoprotein Ib–IX complex: identification of sulfated residues and effect on ligand binding. *Biochemistry* 1994; 33: 946–952.
83 Shen Y, Romo GM, Dong JF et al. Requirement of leucine-rich repeats of glycoprotein (GP) Ib alpha for shear-dependent and static binding of von Willebrand factor to the platelet membrane GP Ib–IX–V complex. *Blood* 2000; 95: 903–910.
84 Miller JL, Lyle VA, Cunningham D. Mutation of leucine-57 to phenylalanine in a platelet glycoprotein Ibα leucine tandem repeat occurring in patients with an autosomal dominant variant of Bernard–Soulier disease. *Blood* 1992; 79: 439–445.
85 Ware J, Russell SR, Marchese P et al. Point mutation in a leucine-rich repeat of platelet glycoprotein Ibα resulting in the Bernard–Soulier syndrome. *J Clin Invest* 1993; 92: 1213–1220.
86 Du X, Ginsberg MH. Integrin $\alpha_{IIb}\beta_3$ and platelet function. *Thromb Haemost* 1997; 78: 96–100.
87 Calvete JJ. Platelet integrin GPIIb/IIIa: Structure–function correlations. An update and lessons from other integrins. *Proc Soc Exp Biol Med* 1999; 222: 29–38.
88 Loftus JC, Halloran CE, Ginsberg MH, Feigen LP, Zablocki JA, Smith JW. The amino-terminal one-third of α_{IIb} defines the ligand recognition specificity of integrin $\alpha_{IIb}\beta_3$. *J Biol Chem* 1996; 271: 2033–2039.
89 Kamata T, Irie A, Tokuhira M, Takada Y. Critical residues of integrin α_{IIb} subunit for binding of $\alpha_{IIb}\beta_3$ (glycoprotein IIb–IIIa) to fibrinogen and ligand-mimetic antibodies. *J Biol Chem* 1996; 271: 18610–18615.
90 Honda S, Tomiyama Y, Shiraga M et al. A two-amino acid insertion in the Cys146–Cys167 loop of aIIb subunit is associated with a variant of Glanzmann thrombasthenia: critical role of Asp 163 in ligand binding. *J Clin Invest* 1998; 102: 1183–1192.
91 Kaushansky K, Lok S, Holly RD et al. Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. *Nature* 1994; 369: 568–571.
92 Kojima H, Hamazaki Y, Nagata Y, Todokoro K, Nagasawa T, Abe T. Modulation of platelet activation in vitro by thrombopoietin. *Thromb Haemost* 1995; 74: 1541–1545.
93 Van Os E, Wu YP, Pouwels JG et al. Thrombopoietin increases platelet adhesion under flow conditions and decreases rolling. *Thromb Haemost* 2001; in press.
94 Nieuwenhuis HK, Sakariassen KS, Houdijk WPM, Nievelstein PFEM, Sixma JJ. Deficiency of platelet membrane glycoprotein Ia is associated with a decreased platelet adhesion to

Cambridge Books Online © Cambridge University Press, 2009

subendothelium. A defect in platelet spreading. *Blood* 1986; 68: 692–695.

95 Santoro SA. Identification of a 160 000 dalton platelet membrane protein that mediates the initial divalent cation-dependent adhesion to collagen. *Cell* 1986; 46: 913–920.

96 Kunicki TJ, Kritzik M, Annis DS, Nugent DJ. Hereditary variation in platelet integrin $\alpha 2\beta 1$ density is associated with two silent polymorphisms in the $\alpha 2$ gene coding sequence. *Blood* 1997; 89: 1939–1943.

97 Humphries MJ. Integrin structure. *Biochem Soc Trans* 2000; 28: 311–339.

98 Depraetere H, Wille C, Gansemans Y et al. The integrin $\alpha 2\beta 1$ (GPIa/IIa)-I-domain inhibits platelet–collagen interaction. *Thromb Haemost* 1997; 77: 981–985.

99 Leahy DJ, Aukhil I, Erickson HP. 2.0–Å crystal structure of a four domain segment of human fibronectin encompassing the RGD loop and synergy region. *Cell* 1996; 84: 155–164.

100 Baneres JL Roquet F, Green M, LeCalvez H, Parello J. The cation-binding domain from the a-subunit of $\alpha 5\beta 1$ is a minimal domain for fibronectin recognition. *J Biol Chem* 1998; 273: 24744–24753.

101 Di Paola J, Federici AB, Mannucci PM et al. Low platelet alpha2beta1 levels in type I von Willebrand disease correlate with impaired platelet function in a high shear stress system. *Blood* 1999; 93: 3578–3582.

102 Roest M, Sixma JJ, Wu YP et al. Platelet adhesion to collagen in healthy volunteers is influenced by variation of both $\alpha 2\beta 1$ density and von Willebrand factor. *Blood* 2000; 96: 1433–1437.

103 Afshar-Kharghan V, Li CQ, Khoshnevis-Asl M, Lopez JA. Kozak sequence polymorphism of the glycoprotein Ibα gene is a major determinant of the plasma membrane levels of the platelet GPIb–IX–V complex. *Blood* 1999; 94: 186–191.

104 Kunicki TJ, Honda S, Dawson B, Honda Y, Ruan C, Aster RH. The P1a alloantigen system is a sensitive indicator of the structural integrity of the amino-terminal domain of the human $\beta 3$ subunit. *Blood Cells Mol Dis* 1995; 21: 131–141.

105 Jansson JH, Nilsson TK, Johnson O. von Willebrand factor in plasma: a novel risk factor for recurrent myocardial infarction and death. *Br Heart J* 1991; 66: 351–355.

106 Koster T, Blann AD, Briet E, Vandenbroucke JP, Rosendaal FR. Role of clotting factor VIII in effect of von Willebrand factor on occurrence of deep-vein thrombosis. *Lancet* 1995; 345: 152–155.

107 Uitterlinden AG, Burger H, Huang Q et al. Relations of alleles of the collagen type Ia gene to bone density and the risk of osteoporotic fractures in postmenopausal woman. *N Eng J Med*. 1998; 338: 1016–1021.

108 Thompson SG, Kienast J, Pyke SD, Haverkate F, van der Loo JC. Hemostatic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. *N Eng J Med* 1995; 332: 635–641.

109 Linenberger ML, Kindelan J, Bennett RL, Reiner AP, Cote HC. Fibrinogen Bellingham: a gamma-chain R275C substitution and a beta-promoter polymorphism in a thrombotic member of an asymptomatic family. *Am J Hematol* 2000; 64: 242–250.

110 Caen JP, Castaldi PA, Leclerc JC. Congenital bleeding disorders with long bleeding time and normal platelet count I: Glanzmann's thrombasthenia (report of 15 patients). *Am J Med* 1966; 41: 4–12.

111 Sadler JE, Mannucci PM, Berntorp E et al. Impact, diagnosis and treatment of von Willebrand disease. *Thromb Haemost* 2000; 84: 160–174.

112 Stel HV, Sakariassen KS, de Groot PG, van Mourik JA, Sixma JJ. The von Willebrand factor in the vessel wall mediates platelet adherence. *Blood* 1985; 65: 85–90.

113 Weiss HJ, Hawiger J, Ruggeri ZM, Turitto VT, Thiagarajan P, Hoffmann T. Fibrinogen-independent platelet adhesion and thrombus formation on subendothelium mediated by glycoprotein IIb–IIIa complex at high shear rate. *J Clin Invest* 1989; 83: 288–297.

114 Sadler JE, Matsushita T, Dong Z, Tuley EA, Westfield LA. Molecular mechanism and classification of von Willebrand disease. *Thromb Haemost* 1995; 74: 161–166.

115 Fressinaud E, Baruch D, Rothschild C, Baumgartner HR, Meyer D. Platelet von Willebrand factor: evidence for its involvement in platelet adhesion to collagen. *Blood* 1987; 70: 1214–1217.

116 d'Allesio P, Zwaginga JJ, de Boer HC et al. Platelet adhesion to collagen in subtypes of type I von Willebrand's disease is dependent on platelet von Willebrand factor. *Thromb Haemost* 1990; 64: 227–231.

117 Wu YP, van Breugel HFI, Lankhof H et al. Platelet adhesion and von Willebrand Factor. *Arterioscl Thromb Vasc Biol* 1996; 16: 611–620.

118 de Groot PG, Federici AB, de Boer HC, d'Alessio P, Mannucci PM, Sixma JJ. Von Willebrand factor synthesized by endothelial cells from a patient with type IIB von Willebrand disease supports platelet adhesion normally, but has an increased affinity for platelets. *Proc Natl Acad Sci USA* 1989; 86: 3793–3797.

119 Nichols WC, Ginsburg D. von Willebrand disease. *Medicine* 1997; 76: 1–20.

120 Hillery CA, Mancuso DJ, Sadler EJ et al. Type 2M von Willebrand disease: F606I and I662F mutations in the glycoprotein Ib binding domain selectively impair ristocetin- but not botrocetin-mediated binding of von Willebrand factor to platelets. *Blood* 1998; 91: 1572–1581.

121 Weiss HJ, Meyer D, Rabinowitz R et al. Pseudo-von Willebrand's disease. An intrinsic platelet defect with aggregation by unmodified human factor VIII / Willebrand factor and enhanced adsorption of its high-molecular-weight multimers. *N Engl J Med* 1982; 306: 326–333.

122 Russell SD, Roth GJ. Pseudo-von Willebrand disease: a mutation in the platelet glycoprotein Ib alpha gene associated with a hyperactive surface receptor. *Blood* 1993; 81: 1787–1791.

123 Veyradier A, Jenkins SP, Fressinaud E, Meyer D. Acquired von Willebrand syndrome: from pathophysiology to management. *Thromb Haemost* 2000; 84: 175–182.

Cambridge Books Online © Cambridge University Press, 2009

124 van Genderen PJ, Vink T, Michiels JJ, van 't Veer MB, Sixma JJ, van Vliet HH. Acquired von Willebrand disease caused by an autoantibody selectively inhibiting the binding of von Willebrand factor to collagen. *Blood* 1994; 84: 3378–3384.

125 Kunicki TJ, Nurden AT, Pidard D, Russell NR, Caen JP. Characterization of human platelet glycoprotein antigens giving rise to individual immunoprecipitates in crossed-immunoelectrophoresis. *Blood* 1981; 58: 1190–1197.

126 Phillips DR, Charo IF, Scarborough RM. GPIIb-IIIa: the responsive integrin. *Cell* 1991; 65: 359–362.

127 Sakariassen KS, Nievelstein PF, Coller BS, Sixma JJ. The role of platelet membrane glycoproteins Ib and IIb-IIIa in platelet adherence to human artery subendothelium. *Br J Haematol* 1986; 63: 681–691.

128 Roth GJ. Developing relationship: arterial platelet adhesion, glycoprotein Ib, and leucine-rich glycoproteins. *Blood* 1991; 77: 5–19.

129 Nievelstein PFEM, Sixma JJ, Ottenhof-Rovers M, Wijnne HJ, de Groot PG, Banga JD. Platelet adhesion and aggregate formation in diabetes mellitus type I under flow conditions. *Diabetes* 1991; 40: 1410–1417.

130 Ni H, Denis CV, Subbarao S et al. Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. *J Clin Invest* 2000; 106: 385–392.

131 Kyriakides TR Leach KJ, Hoffman AS, Ratner BD, Bornstein P. Mice that lack thrombospondin-2 display connective tissue abnormalities that are associated with disordered collagen fibrillogenesis, and increased vascular density, and a bleeding diathesis. *J Cell Biol* 1998; 140: 419–430.

132 Agbanyo FR, Sixma JJ, de Groot PG, Languino LR, Plow EF. Thrombospondin-platelet interactions: Role of divalent cations, wall shear rate and platelet membrane glycoproteins. *J Clin Invest* 1993; 92: 288–296.

133 van Zanten GH, de Graaf S, Slootweg PJ, Heynen FG, de Groot PG, Sixma JJ. Increased platelet deposition on atherosclerotic coronary arteries. *J Clin Invest* 1994; 93: 615–632.

134 Roux SP, Sakariassen KS, Turitti VT, Baumgartner HR. Effect of aspirin and epinephrine on experimentally induced thrombogenesis in dogs: a parallelism between in vivo and ex vivo thrombosis models. *Arterioscler Thromb* 1991; 11: 1182–1191.

135 Strony J, Beaudoin A, Brands D, Adelman B. Analysis of shear stress and hemodynamic factors in a model of coronary artery stenosis and thrombosis. *Am J Physiol* 1993; 265: H1787–H1796.

136 Slack SM, Cui Yw, Turitto VT. The effects of blood flow on coagulation and thrombosis. *Thromb Haemost* 1993; 70: 129–134.

137 Van Breugel HHFI. Hemorheology and its role in blood platelet adhesion under flow conditions. Thesis, 1989.

Cambridge Books Online © Cambridge University Press, 2009

22

The platelet shape change

Adrian R.L. Gear and Renata K. Polanowska-Grabowska

Department of Biochemistry and Molecular Genetics
University of Virginia School of Medicine
Charlottesville, USA

Introduction

Blood platelets respond to many stimuli by changing shape from their resting, normal discoid form into more rounded structures possessing blebs and pseudopodia[1–5]. These morphological transitions are generally referred to as the platelet '**shape change' (SC)**. As described earlier in Chapters 2 and 3, platelets are subcellular fragments derived from megakaryocytes and circulate in the blood as small anucleated ovoid discs, with average dimensions of about 3.0×0.5 μm and volumes of 6–8 fl[6,7]. The surfaces of resting platelets are almost featureless except for indentations or pits that represent entrances into the open-canalicular system (OCS) illustrated in a freeze–fracture micrograph (Fig. 22.1). Platelets exhibit significant heterogeneity in both size, density and biochemical composition[8–12], with a corresponding heterogeneity in aggregation and adhesion characteristics[8,13–15]. Shape-change behaviour is also heterogeneous, such that some platelets will remain discoid while neighbouring cells will have undergone significant morphological changes[16].

The transformations in platelet morphology associated with the SC represent highly sensitive indices of platelet activation. They are generally considered to be the first measurable physiological responses after activation by specific platelet agonists such as ADP or thrombin, and to begin before significant aggregation occurs. Platelet SC is usually characterized morphologically by spheration and contraction of the cell, cytoskeletal rearrangements, folding or ruffling of the surface membrane, and formation of pseudopodia[17,18]. An example of the major changes from the resting discoid shape to more rounded forms possessing multiple pseudopodia is illustrated in Fig. 22.2. Changes in morphology also occur following platelet contact with the foreign particles or surfaces, centrifugation, by exposure to heat, cold, calcium chelation such as with EDTA or EGTA, and by depressed energy metabolism[19–22].

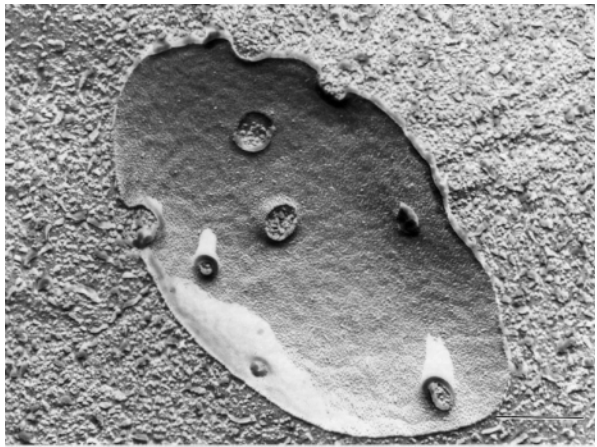
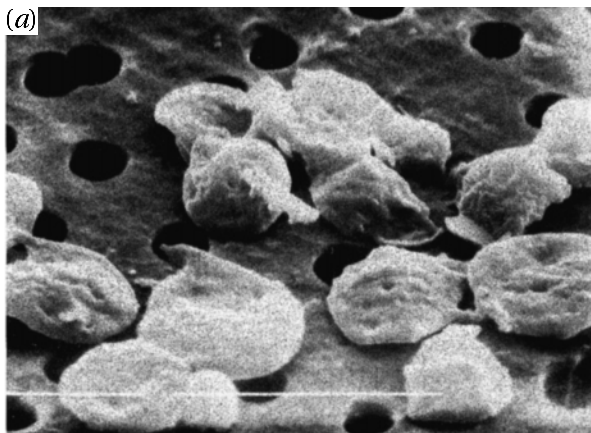


Fig. 22.1. A resting blood platelet. The nearly smooth surface of a blood platelet visualized by freeze- fracture electron microscopy[187] and rapid spray freezing to avoid chemical fixation[38]. The surface 'pits' represent entrances to the open canalicular system or OCS[7]. The bar represents 0.5 μm.

As mentioned above, the initial phases of the SC precede aggregation and can even occur in the absence of any aggregation as seen in thrombasthenic platelets which lack the $\alpha_{IIB}\beta_3$ integrin[23]. Transformation from a discoid shape to one possessing many pseudopodia dramatically increases the external platelet surface area, facilitating aggregation. The details and interrelationships of the intracellular signalling pathways involved in the platelet SC and reorganization of the cytoskeleton are not yet fully understood. An overview of



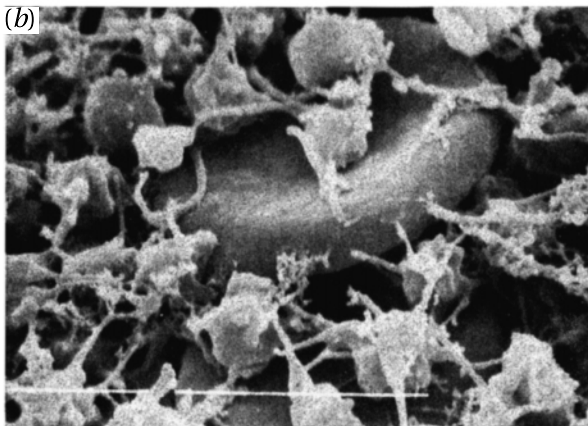


Fig. 22.2. The platelet shape change (SC). Scanning electron micrographs (SEM) reveal the dramatic transformation of the surface morphology following stimulation by the important agonist ADP. Upper figure, resting (control) platelets; Lower figure, after 10 s exposure to ADP (10 μM) at 37 °C. The formation of long, actin-rich pseudopodia is characteristic of the SC and can proceed very rapidly as demonstrated in Fig. 22.4. Platelets were activated in dilute suspensions to minimize aggregation[19]. The middle bar on the bottom represents 5 μm.

current research into signal-transduction events occurring during the SC, including calcium-dependent and independent mechanisms, will be presented on pp. 324–329. Overall, the rapid and dramatic changes in surface morphology characteristic of the SC have fascinated investigators interested in the fundamental mechanisms of platelet activation and for understanding links to normal and abnormal haemostasis.

Definitions of the platelet 'shape change'

An issue in SC studies, whether for basic research or clinical purposes, concerns the meaning of the term 'shape change'. Therefore, it is important to be aware of the varied definitions and procedures that have been used. These are summarized below and will be discussed in more detail on pp. 324–326.

(a) Alterations in the light-scattering properties of platelet suspensions, reflecting transitions from the discoid shape of resting platelets to more spherical forms possessing blebs and pseudopodia.
(b) Alterations in platelet morphology visualized by a variety of microscopic procedures, including light microscopy, as well as by scanning, transmission and freeze–fracture electron microscopy.
(c) Alterations in platelet volume or size, normally detected by resistive-particle counters, but also by flow cytometry.
(d) Alterations in the surface exposure of platelet antigens usually detected by immunological means and flow cytometry.

Events associated with the normal platelet shape change

The 'optical shape change'

The original definition of the platelet SC stems from observations that, when platelet agonists are added to stirred, platelet-rich plasma (PRP), there is very often a brief, initial **decrease** in light transmission (increase in optical density) before the major **increase** associated with platelet aggregation[1]. The early decrease in transmission usually lasts about 10 to 20 s and amounts to about 10% of the total change caused by aggregation. The diagrammatic tracing in Fig. 22.3 represents a 'classical' form when an intermediate dose of ADP elicits the initial SC, followed by two waves of aggregation. The first wave has been termed 'primary aggregation' and the subsequent one 'secondary aggregation'. Higher doses of ADP or thrombin will elicit only a single phase of aggregation after the initial SC. Low doses of agonist may induce the SC and only a brief reversible phase of aggregation.

Events sensed by light scattering or optical devices, whether as the SC reflected by the initial decrease in light transmission (p. 321) or the subsequent decreases indicating platelet aggregation, need to be correlated with other observational techniques. Thus, alterations in platelet morphology (p. 322), volume (p. 324), or new epitope expo-

Cambridge Books Online © Cambridge University Press, 2009

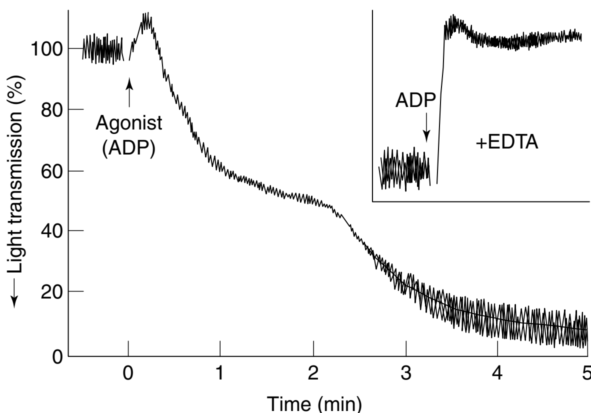


Fig. 22.3. The platelet SC assessed optically. The trace is a representation of a typical progression of changes in light transmission through platelet rich plasma following the addition of threshold levels of ADP (usually 2 to 5 μM). Three phases may occur; an initial 'shape change' (SC) lasting about 20 s, followed by two phases of aggregation. High doses of ADP or thrombin induce the SC and only one aggregation phase. Standard procedures usually involve setting the transmission through platelet poor plasma (PPP) at 0%, and for platelet rich plasma (PRP) at 100%. Samples are stirred at about 1000 rpm to provide optimal sensitivity for detecting the 'SC' and to ensure platelet aggregation by efficient particle collision[28]. The inset shows SC occurring in the absence of aggregation. PRP is treated with EDTA (typically 2–4 mM) to chelate calcium and diluted 1 to 4 to minimize any tendency to aggregation[1,19,38] and the decrease in transmission is typical of stimulation by ADP.

sure (p. 323), provide important complementary information on SC kinetics and characteristics. An indication of this need for multiparameter assessment of the SC comes from the fact that single-particle disappearance, representing platelet micro-aggregation, is essentially complete before the peak (Fig. 22.3) in the optical SC at about 10–20 s[24], and does not correlate with the aggregation kinetics sensed by the optical traces[25]. Thus, the optical SC is compromised by concurrent aggregation. In a similar vein, the later aggregation phases (30 s – 5 min) need to be distinguished from the very rapid (<5 s) formation of micro-aggregates detected as the loss of single platelets. The capture angle of the photometers used to detect transmitted or scattered light can also impact on whether or not an initial decrease in transmission is seen[26].

Light-scattering devices provide additional information about platelet shape. Thus, the width of the oscillations or 'noise' of the transmitted light prior to adding agonist is normally large when the platelets are discoid, and becomes much narrower when sphering occurs[24,27]. The schematic tracing in Fig. 22.3 provides an example of this 'noise' seen in platelet suspensions subjected to shear forces caused by stirring in the small tubes used in standard aggregometers. In addition, when stirring is stopped, light transmission decreases in proportion to the particle axial ratio which reflects platelet discoid shape[26]. These effects contribute to the characteristic 'swirling' of fresh platelet preparations. A review by Frojmovic[28] presents the varied optical parameters employed to describe both the SC and aggregation phases of platelet function. In addition, the use of polarized light and assessing light scattering changes at 90° to the incident light can yield valuable information about the SC[5]. An in-depth study by Hantgan involved 90° light scattering to define the kinetics of the optical SC induced by ADP, and showed that only about 3 s was needed to reach one-half maximal change[29].

Use of calcium chelating agents to study the SC

The inset to Fig. 22.3 shows the optical SC detected in the presence of EDTA at concentrations sufficient to block platelet aggregation[19,30]. Measurements can also be made in very dilute platelet suspensions to minimize any further chance of aggregation, with the result that an agonist still causes the decrease in light transmission without any major subsequent increases. Kinetics derived from such traces are similar to those obtained by Hantgan[29], with $t^{1/2}$ values of <5 s. Such strategies to 'isolate' the optical SC have been effective in many studies and most recently for dissection of specific, signal-transduction events linked to induction of the SC[31,32]. EDTA may, however, be deleterious since it can also elicit significant platelet swelling[19] and sphering[22,33–35].

Morphological shape changes

The ability of agonists such as ADP and thrombin to alter platelet shape as visualized by light and electron microscopy was reported in the 1960s at the same time that light scattering was used as an easy way to sense the SC. Platelet rounding was noted as well as the appearance of multiple pseudopodia[3–5,27,36]. The study by White[33,36] using transmission electron microscopy provided the first important observations on details of external morphology and internal events during the ADP-induced SC. Multiple long pseudopodia appeared by 10 s after adding ADP and central grouping of internal organelles was noted.

An analysis of even earlier morphological changes by scanning electron microcopy (SEM) revealed that 50% of platelets lost their discoid shape by 0.5 s after platelet exposure to ADP or thrombin and that about 60% of cells had formed multiple pseudopodia by 1.7 s[16]. Platelets were

Cambridge Books Online © Cambridge University Press, 2009

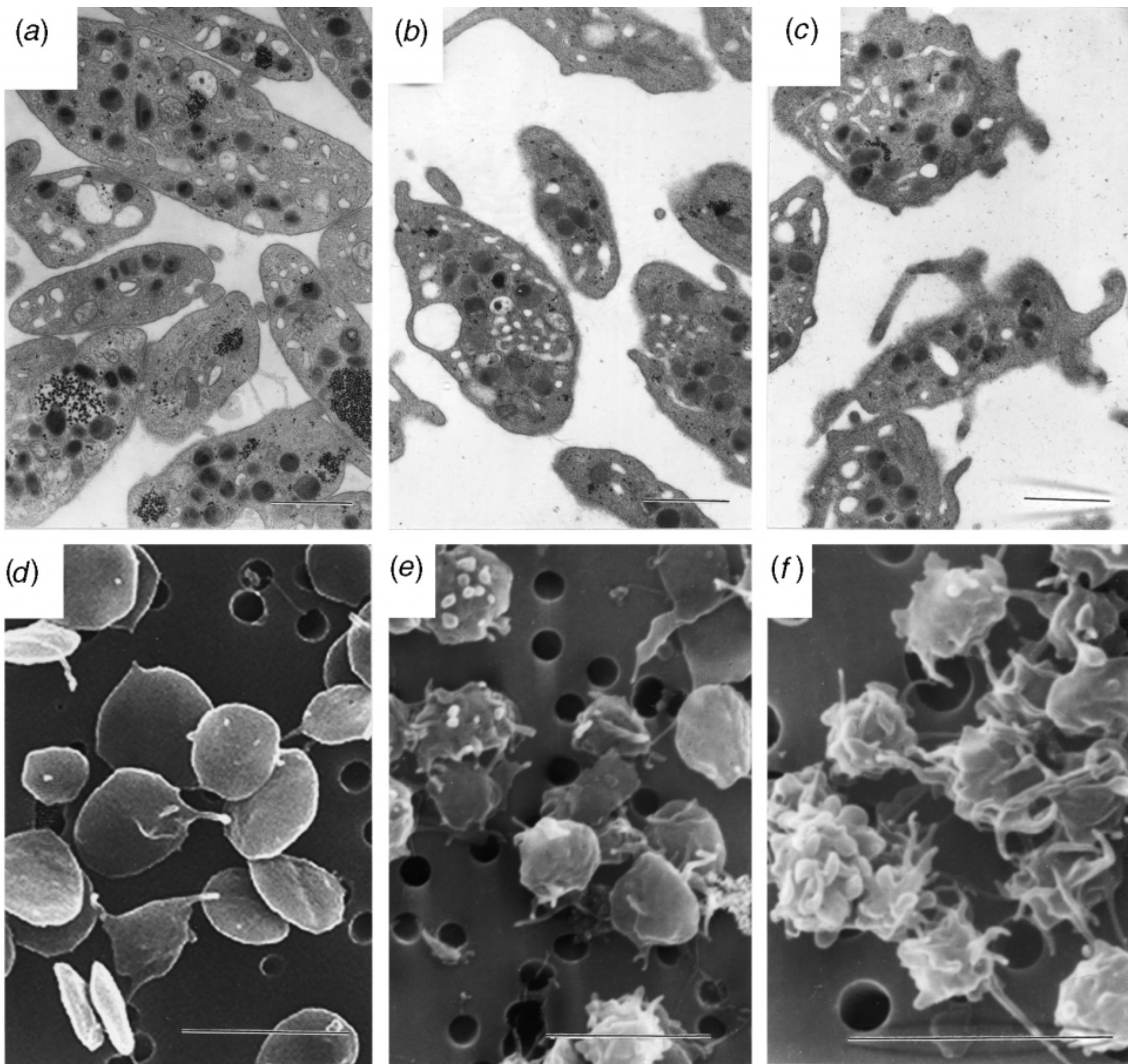


Fig. 22.4. The speed of the platelet SC. Platelets previously warmed for 15 min at 37 °C were exposed to ADP (10 μM for panels (*a*)–(*c*)) or to thrombin (1 U/ml for panels (*d*)–(*f*)) in a quenched-flow system under arterial shear forces before quenching with glutaraldehyde and processing for transmission electron microscopy (TEM) or SEM (Gear, 1984). The TEM images shown are: control resting platelets (*a*), cells reacted with ADP for 0.5 s (*b*), and for 1.25 s (*c*): SEM images; control platelets (*d*), cells reacted with thrombin for 0.5 s (*e*) and for 1.7 s (*f*) (Magnifications are provided on the figures as bars; 1 μm for (*a*)–(*c*), 5 μm for (*d*)–(*f*).

exposed to arterial shear forces and use of rapid quenched-flow methods enables study of second and to subsecond kinetics[19,37]. Results from platelets stimulated for 0.5 and 1.25 s by ADP under flow conditions and examined by transmission electron microscopy (TEM) are illustrated in Fig. 22.4(*a*)–(*c*) and by SEM for thrombin-activated platelets in Fig. 22.4(*d*)–(*f*). Similar kinetics are seen for either agonist. Freeze–fracture electron microscopy of spray–frozen platelets activated for about 1 s by ADP provides an alternative fixation-free approach for studying the platelet SC under arterial shear forces[38]. The image presented in Fig. 22.5 can be compared to the control platelet seen in Fig. 22.1 subjected to the same arterial shear forces in the continuous-flow pumping system.

Cambridge Books Online © Cambridge University Press, 2009

Fig. 22.5. ADP-activated platelets visualized by freeze–fracture electron microscopy. Cells were reacted to ADP for about 1 s and quenched by spray freezing . The image reveals the presence of 'blebs' compared to the smooth surface seen with control, spray–frozen cells (Fig. 22.1) (Magnification bar is 1 μm.

Volume increases

Shape change can also be sensed by changes in platelet volume. The ability of platelet aggregating agents such as ADP to increase cell volume by about 20% was reported in the 1960s[3,39,40]. Born then questioned the validity of measurements based on resistive-particle counting[1]. He used centrifugation procedures in the presence of EDTA and a version of the hematocrit to suggest that the optical SC was not associated with an increase in platelet volume. However, EDTA itself can induce platelet swelling and sphering[33,34,39,41]. These effects, plus the stress of centrifugal forces in causing platelet swelling, serve to mask the ability of ADP to cause an increase in cell volume[19]. Other observations consistent with agonist- induced swelling are decrease in cell density[19] and an increase in Na^+ content[42–44]. Direct morphometric estimation of ADP- activated platelets also supports the volume increases sensed by resistive-particle counters[45].

This brief background serves to underpin present confidence in volume measurements as a useful alternative approach to sensing the platelet SC. A useful description of some general aspects by Jackson and Carter[46] and Jagroop et al.[47] provide valuable correlations between agonist-induced volume increases and platelet shape directly observed by SEM. However, to study the kinetics of agonist-induced increases rather than long-term or static situations, continuous-flow systems are required[37,48]. ADP and thrombin can induce major increases by 0.5 to 1.0 s, and by 10 s, net increases may be 10 to 20% above resting platelet volumes of 6 to about 7.7 fl. The initial speeds of reaction (<0.5 s) approach those needed for efficient platelet function under arterial-flow conditions[49]. In conclusion, platelet-volume measurements now offer an important extra dimension for assessing the platelet SC. A schematic summary of the initial kinetics of the volume increase occurring during the SC, as well as major morphological events, is presented in Fig. 22.6.

The SC defined by exposure of 'new' platelet surface structures and antigens

Activation of platelets by some agonists or by shear stresses can alter platelet morphology and expose new features or molecules on the platelet surface without triggering detection of the SC defined by the criteria presented above

Cambridge Books Online © Cambridge University Press, 2009

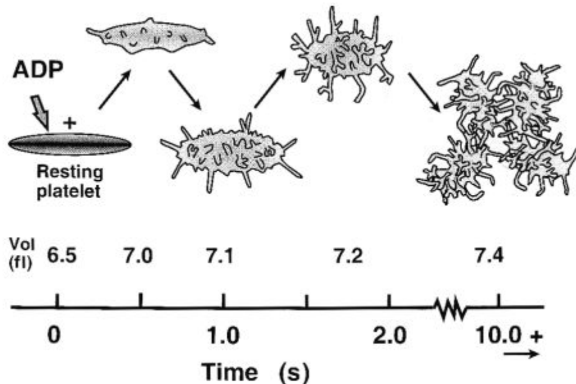


Fig. 22.6. Summary of initial SC events. The scheme illustrates the kinetics of SC visualized by SEM and TEM (Fig. 22.4), as well as reflected in the volume increases caused by agonists such as ADP. The absolute volumes and kinetics of the changes in volume are as published[16,19,47].

(pp. 320–323). Such changes may represent a significant step in the initiation of platelet function, with important overall consequences for hemostasis. For example, the 'weak' agonist epinephrine characteristically fails to elicit an optical SC or increase in platelet volume[19,37]. Aggregation occurs only in the presence of very low levels of a primary agonist such as ADP[50], with little evidence of a SC detected optically[51]. Recent studies have now shown that other weak agonists such as chemokines stimulate platelet function and cause the formation of 'blebs' on the platelet surface, with epinephrine behaving similarly[52]. Of particular interest was the finding that the platelet α-granule protein CD62P (P-selectin) is exposed as detected by flow cytometry even though there was no evidence for dense granule secretion.

Flow cytometry has been used for many years to detect markers of platelet activation such as the activated conformations of the fibrinogen receptor, the $\alpha_{IIB}\beta_3$ integrin[53]. A review article by the European group on clinical cell analysis[54] provides a valuable perspective on the expanding capabilities of flow cytometry with regard to platelet function. The SC represents an important initial phase of function and availability of well-defined antibodies will provide useful ways to assess platelet activation not just for ex vivo investigations, but also for in vivo monitoring of circulating platelet status in different clinical situations[55]. P-selectin is just one of a number of activation-dependent markers; others include procoagulant anionic phospholipids and the $\alpha_{IIB}\beta_3$ integrin.

Mechanisms and regulation of shape change – intracellular signalling

Less is known about the biochemical events associated with the SC compared to subsequent phases of platelet function[56,57]. An early study highlighted the rapid phosphorylation of myosin light chains in ADP-stimulated platelets, and how this parallelled the kinetics of the SC assessed optically as well as by scanning electron microscopy[58]. Although initial interest was mostly on calcium and myosin light chain phosphorylation in relation to the SC, more recent studies have revealed other participants such as small GTP-binding proteins including Rho, Rac, and Cdc42 and the p160 Rho-associated kinase (Rho-kinase). In addition, new information is helping elucidate the roles of many proteins and factors involved in regulating platelet shape and structure, the dynamics of the actin cytoskeleton, membrane skeleton and microtubules. The aim of this section is to provide a sense of what is now known about signalling events involved in the SC as well as questions for the future.

Intracellular calcium

Major increases in cytosolic calcium levels $[Ca^{2+}]_i$ usually occur during activation of platelet function[59]. When platelets are stimulated by many agonists such as ADP or thrombin, cytosolic calcium levels rapidly increase (<5 s), associated with both a large influx through the plasma membrane and increased mobilization of calcium from intracellular stores[59–61]. However, SC-induced by certain platelet agonists can be mediated through pathways that does not require elevation of $[Ca^{2+}]_i$[62,63]. Agonists that have been shown to induce this calcium-independent pathway of SC include the heptapeptide ligand of the thrombin receptor, YFLLRNP, low concentrations of thrombin[57,64], the endoperoxide analogues U46619 and U44069[31], and mildly oxidized, low-density lipoprotein (mox-LDL)[65]. Consistent with this is the observation that stimulation of G_q-deficient mouse platelets by various stimuli fails to activate phospholipase C and alter $[Ca^{2+}]_i$ levels, but the platelets still undergo SC in the absence of aggregation and degranulation[66]. In addition, the initial rate and the onset of the SC are markedly delayed in platelets exposed to the intracellular calcium chelator BAPTA-AM[67].

Myosin light chain phosphorylation

A strong correlation exists between phosphorylation of the myosin light chain (MLC) and the initiation of SC[58,68], and the time course of MLC phosphorylation parallels that of

Cambridge Books Online © Cambridge University Press, 2009

association of myosin with the actin cytoskeleton[67,69]. Phosphorylated myosin interacts mainly with central actin filaments in platelets, and the myosin-actin complex has been suggested to be involved in the granule centralization process[17,69]. The 20 kDa MLC is phosphorylated during SC induced by ADP, serotonin, vasopressin, PAF, collagen, A23187, endoperoxide analogues and by chilling[58,68,70–72]. Epinephrine by itself does not stimulate MLC phosphorylation or the typical SC from discoid to spheroid platelets[73], but in combination with ADP contributes strongly to SC and volume increases[16,50].

MLC phosphorylation is very rapid ($t^{1/2}$ about 1.5 s) and precedes the induction of the optical SC ($t^{1/2}$ about 2.5 s). Dephosphorylation occurs soon after platelet activation by ADP or thrombin and does not parallel reversal of platelet shape back to a discoid form[58,72,74,75]. Therefore, myosin phosphorylation may not be required for the maintenance of the SC response. Net phosphorylation represents a balance between the activity of the Ca^{2+}/calmodulin dependent MLC kinase (MLCK) and MLC-phosphatase (MLCP) and there is now strong evidence that MLC phosphorylation can be induced independently of changes in cytosolic Ca^{2+} concentration[76]. This theme will be developed below.

Rho kinases and the SC

Recent studies have revealed that Rho-kinases, which can be activated by the small guanosine triphosphate (GTP)-binding protein RhoA, directly phosphorylates the 130-kDa myosin-binding, regulatory subunit (MBS) of protein phosphatase 1 (PP1), inhibiting phosphatase activity. The overall effect is to increase MLC-phosphorylation[77,78]. This mechanism for stimulating the SC is independent of any increase in cytosolic $[Ca^{2+}]_i$[31]. Several platelet agonists induced the SC through both Ca^{2+}-sensitive and -insensitive pathways which independently contributed to the SC and MLC phosphorylation[67]. The Ca^{2+}-dependent myosin phosphorylation is very rapid, peaking near 2 s after stimulation with ADP, thrombin or the thromboxane A_2 (TXA_2) analogue, U46619[67]. These kinetics are very similar to earlier evidence for ADP and thrombin stimulation of MLC phosphorylation[8,79]. Experiments with the relatively selective inhibitors Y-27623 and HA 1077 of the $p160^{ROCK}$ or Rho-kinase and with BAPTA-AM, support the conclusion that Rho kinase is involved in stabilizing the phosphorylation of the MLC[80].

In summary, these recent reports highlight the fact that an increase in intracellular calcium is not the only signal involved in stimulating the SC via MLCK. While an important parallel role for Rho-kinases is clearly involved, the relative significance of the two kinase pathways, and possibly others, has yet to be clarified for different platelet stimuli.

Heterotrimeric G-proteins and the SC

Platelets from G_q-deficient mice appear to undergo SC when exposed to TXA_2- receptor agonists, thrombin or collagen, even though they lack phospholipase C activation and formation of inositol phosphates, and are unable to aggregate fully or secrete their granule contents[81]. SC can therefore be mediated by $G_{12/}G_{13}$ activation and as discussed above may involve the Rho/Rho- kinase pathway leading to MLC phosphorylation. In contrast, ADP does not induce SC in G_q-deficient mouse platelets[66,82,83]; and ADP-induced SC of human platelets is insensitive to the Rho-kinase inhibition[32]. Thus, ADP- induced platelet SC can be mediated by the G_q-coupled $P2Y_1$ receptor[31,67,81]. Similarly, activation of G_q-coupled $5HT_{2A}$ receptors by serotonin is sufficient to induce SC[84–86]. Increased levels of cAMP and cGMP may inhibit platelet function via activation of cAMP and cGMP-dependent kinases. However, Klages et al.[81] reported that cAMP but not cGMP analogues inhibit SC and MLC phosphorylation in G_q-deficient mouse platelets activated by the G_{12}/G_{13}-coupled TXA_2 receptor. They propose that SC mediated by Rho/Rho-kinase is specifically inhibited by a cAMP-dependent pathway.

In conclusion, the SC can be activated via several G-protein coupled receptors responding to a variety of different platelet stimuli. G_q- and G_{12}/G_{13}-deficient mouse platelets provide valuable models for studying signalling mechanisms not just for the SC alone, but also for linkages to aggregation and secretion. Soon, other genetically modified animal models may be available to understand even better the complex pathways involved in the SC. Shown in Fig. 22.7 is a basic scheme by which activation of G-protein coupled receptors is believed to elicit the platelet SC. Figure 22.8 illustrates a series of signalling events resulting from G-protein stimulation, including both activating and inhibitory steps as well as some of the important enzymes and regulatory compounds involved in regulating actin and myosin function, leading to the SC.

Protein tyrosine phosphorylation and the SC

Relatively little evidence exists for the SC being directly linked to tyrosine phosphorylation of specific proteins[56]. **Cortactin**, a 77–80 kDa protein which binds to actin filaments, is phosphorylated relatively early (30 s) in thrombasthenic platelets activated with low levels (0.05 U/ml) of thrombin, which induces the SC but no aggregation[81]. A

Cambridge Books Online © Cambridge University Press, 2009

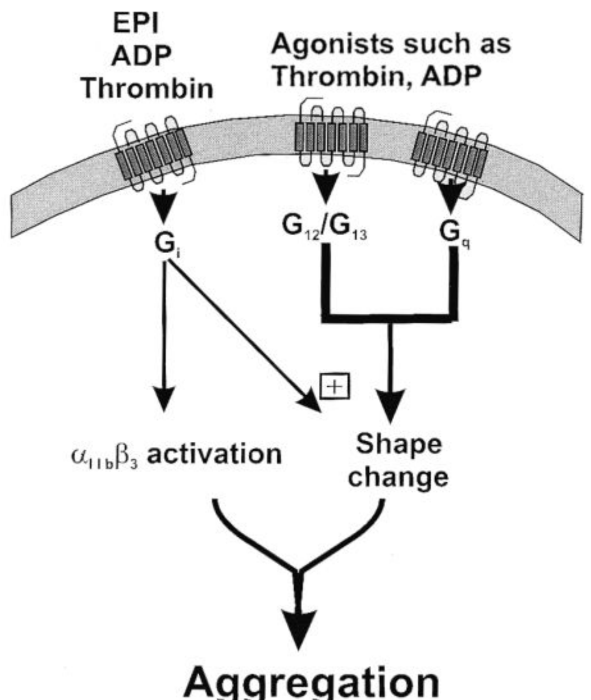


Fig. 22.7. The platelet SC and its linkage to overall platelet function. The main classes of G-protein coupled receptors are indicated[86].

p130 band was also tyrosine phosphorylated early, and a 64 kDa band later (>60 s). The tyrosine kinase Syk is found associated with cortactin by immunoprecipitation experiments[56]. Tyrosine phosphorylation of $p72^{Syk}$ and $p60^{src}$ has also been observed in mouse platelets deficient in G_q, which undergo SC in response to TXA_2, but do not aggregate[81]. Exposure of human platelets to the peptide YFLLRNP, an antagonist of aggregation stimulated by the SFLLNRP thrombin-receptor activating peptide, induces SC without secretion from platelet granule contents, internal Ca^{2+} mobilization, or pleckstrin phosphorylation[64]. The induction of the SC by the YFLLRNP peptide causes protein tyrosine phosphorylation of novel substrate bands at p62, p68, and p130, even in the presence of BAPTA-AM, suggesting an essential role of protein tyrosine kinases for activation of the platelet SC[88]. Mox-LDL induced SC is also accompanied by tyrosine phosphorylation of p62, p68 and p130 and by activation of tyrosine kinase $p72^{Syk}$[65]. In addition, inhibition of ADP-induced SC in aspirin-treated gel-filtered platelets by agents that elevate cAMP and cGMP parallels inhibition of protein tyrosine phosphorylation, suggesting that some of the proteins being tyrosine phosphorylated are important in regulating the ADP-induced SC[11]. A recent study, however, indicates that the ADP- or thrombin-induced SC measured by routine light-transmission methods is not prevented by PP2, an inhibitor of src-family tyrosine kinases[32,89]. On the other hand, broad-spectrum tyrosine kinase inhibitors such as genistein and tyrphostin A23 appear to block SC induced by thrombin or mox-LDL[88,90]. These results support the possibility that unidentified protein tyrosine kinases, unrelated to src-family kinases, may be functionally involved.

Protein kinase C

During SC induced by thrombin, PAF, ADP, vasopressin, concanavalin A, serotonin, thromboxane A_2 analogues, a rapid increase (<2 s) in the phosphorylation of a p47 kDa protein (pleckstrin), a major protein kinase C (PKC) substrate is observed[78,91,92]. Although the non-specific protein kinase inhibitor, staurosporin, strongly blocks pleckstrin phosphorylation and SC induced by ADP, thrombin or U46619, the relatively specific PKC inhibitors Ro 31–8220, GF 109203X and Ro 31–7549 have no effect on agonist-induced SC[67,93,94]. This observation and the demonstration that phorbol esters or diacylglycerol induce maximal phosphorylation of p47 without evoking the rapid spheration of platelets[95], argue against a direct role for PKC in the initial SC.

Role of actin-modulating proteins in the SC

The discoid form of unstimulated platelets is maintained by circumferential bundles of microtubules beneath the plasma membrane, a network of short actin filaments forming a membrane skeleton and an actin gel filament that links the submembrane skeleton and transmembrane proteins[17,18,96]. After activation, actin filaments undergo rapid disassembly and reorganization into new actin structures, including filopodia and lamellipodia, with dynamics closely related to the SC[17,18,97,98]. Thus, actin-modulating proteins must play a critical role in mediating the transmembrane signals that regulate the actin dynamics responsible for the SC. These proteins include myosin, gelsolin, VASP, moesin, adducin and cofilin, with a more complete accounting provided in a recent review of platelet morphology[99]. Early evidence suggested that the circumferential or marginal band of microtubules, which helps maintain resting discoid platelet shape, disappears during the SC[36]. However, the kinetics of this process are not known with regard to the very rapid (<2 s) major changes in platelet shape that have already occurred as assessed optically, or by quenched-flow approaches[1,16,29].

The formation of filopodia and lamellipodia is thought

Cambridge Books Online © Cambridge University Press, 2009

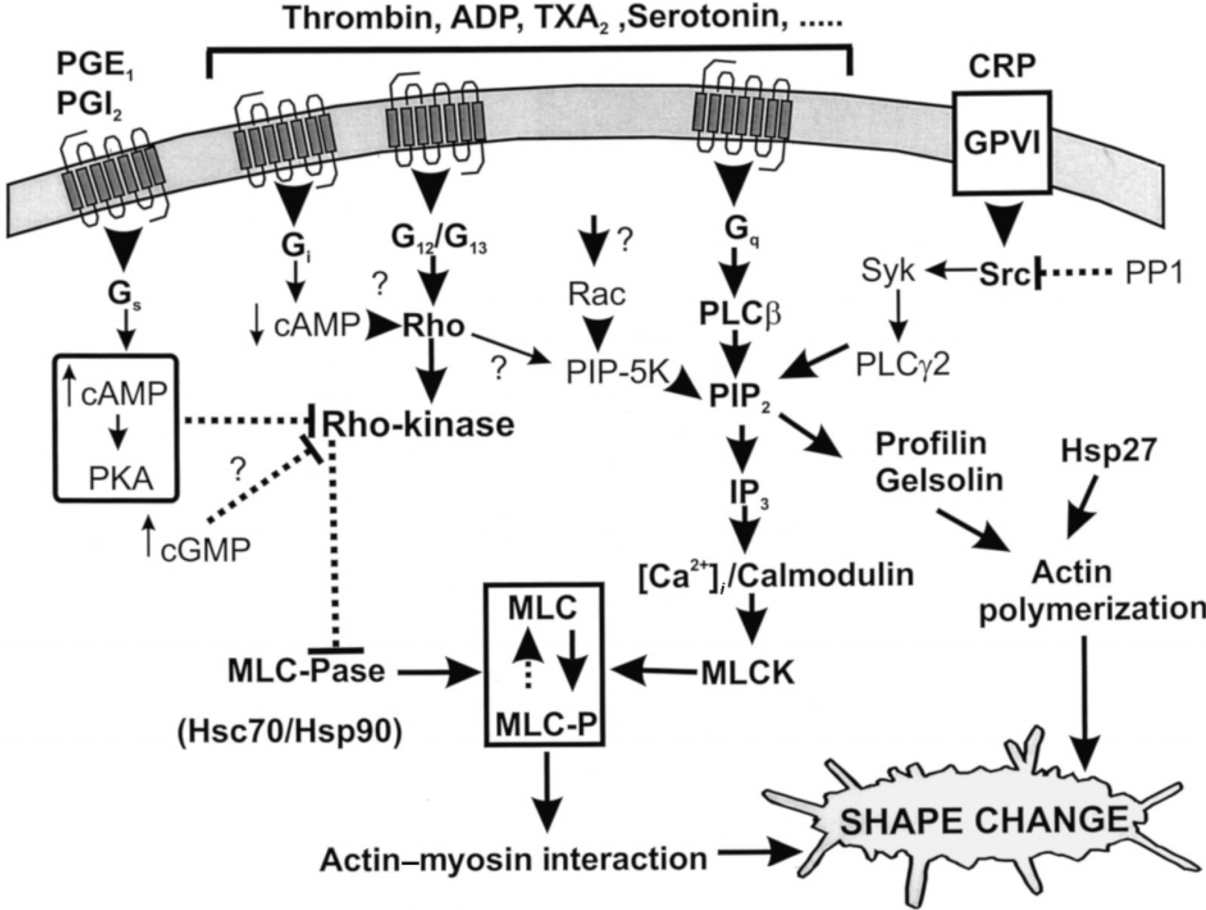


Fig. 22.8. Signalling events during the platelet SC. The main players involved in causing actin polymerization and myosin phosphorylation are shown, together with activation by plasma-membrane receptors, calcium, protein kinases and phosphatases, and heat-shock proteins. The heat-shock proteins, Hsp27 and Hsc70/Hsp90 are also indicated, since there is evidence that they may regulate actin polymerization and the activity of the myosin phosphatase[109,110,116].

to be mediated primarily by **gelsolin**, an abundant actin-filament severing protein. Platelet SC is decreased in gelsolin-deficient mouse platelets and is abnormal in hereditary gelsolin Asp187Asn-related amyloidosis[100,101]. In contrast, the 48–50 kDa vasodilator-stimulated phosphoprotein (**VASP**) is phosphorylated in response to vasodilators and platelet inhibitors that increase cAMP and cGMP levels (prostaglandin E1 (PGE1), forskolin, NO, sodium nitroprusside (SNP)[102–104]) and VASP is believed to inhibit SC by stabilizing actin filaments and preventing their disassembly by gelsolin[105,106]. In VASP-deficient mouse platelets stimulated with collagen, the SC occurs more rapidly than in normal platelets[107]. Earlier studies were consistent with a slow (minutes) phosphorylation of VASP in intact platelets[102]. Recently, it has been shown that platelets from control or VASP-deficient mice exposed to PGE1 are inhibited similarly with regard to platelet function. This result suggests that the inhibitory effects of cAMP are mediated in part via a VASP-dependent mechanism[108], and a clear understanding of the function of VASP and its phosphorylation status in the SC remains to be reached.

Small heat-shock proteins (sHsps) are also involved in regulation of the actin-cytoskeleton[109,110]. In particular, phosphorylation of Hsp27 may play a role in regulating actin polymerization[111–113] and stabilizing the actin cytoskeleton[114]. Recently, Hsp27 has been reported to be phosphorylated by a cGMP-dependent protein kinase (cGK) and thus may be involved in inhibition of platelet responses[115]. In addition, the **large heat-shock proteins**, hsc70 and hsp90, exist in a large signalling complex in resting platelets, which is highly phosphorylated and contains the PP1–M regulatory and catalytic subunits[116]. The roles of this complex in the SC remain to be established.

Cambridge Books Online © Cambridge University Press, 2009

Moesin is a member of the ezrin/radixin/moesin (ERM) family of proteins that acts as a linker between the plasma membrane and actin cytoskeleton and is involved in the formation and stabilization of filopodia[117]. Thrombin stimulation induces a transient increase in moesin phosphorylation at Thr558[118] that correlates with its F-actin binding activity[119]. Phosphorylated moesin binds F-actin only in the presence of phosphoinositides such as $PI(4,5)P_2$. Following thrombin activation, the cytosolic moesin redistributes to the membrane skeleton and co-localizes with actin filaments in newly-formed filopodia and lamellipodia[120]. Moesin is also required for actin assembly mediated by Rho family GTPases[121]. Recently, it has been shown that lysophosphatidic acid (LPA) induces MLC and moesin phosphorylation in a Ca^{2+}- independent manner via Rho/Rho-kinase[122]. However, observations that the moesin-gene-knockout does not affect platelet aggregation[123] might question significance of a critical role for moesin in platelet function.

Adducin, a membrane-skeleton protein, stimulates the binding of spectrin to actin filaments[124]. There is also strong evidence that Rho-kinase and myosin phosphatase (PP1–M) can regulate the F-actin binding activity of adducin through its phosphorylation, promoting the assembly of the spectrin-F-actin network[125,126]. Adducin is also phosphorylated by PKA and PKC[127,128].

Cofilin is an 18 kDa actin-binding protein that can bind to F-actin and depolymerize it[129]. In resting platelets cofilin is phosphorylated and localized to the cytoplasm, but after activation becomes dephosphorylated and translocates to the plasma membrane, complexed with F-actin[130]. The actin-binding and depolymerizing activity of cofilin can be regulated by phosphatidylinositol 4-phosphate (PIP) and 4,5-bisphosphate (PIP_2)[131]. Dephosphorylation kinetics and correlation with the onset of SC have not yet been fully studied. An inhibitor of serine/threonine protein phosphatases, okadaic acid (OA), that causes a dramatic change in platelet morphology[132,133], also blocks dephosphorylation of cofilin in T lymphocytes[134,135]. Since dephosphorylation of cofilin activates its actin-depolymerizing function, activation of serine/threonine protein phosphatases such as PP1 may be involved in the dynamic rearrangement of the actin cytoskeleton.

Kinetics of platelet shape change with regard to signalling

As presented above, the major morphological changes are nearly complete within 2 s after platelet activation by ADP or thrombin[16]. The rapid increases in cytosolic free Ca^{2+} maximal within 2 to 3 s accompany the morphological changes in platelets[60,61]. ADP induces significant increases in MLC phosphorylation by 0.3 s, that reaches a maximum at 3 s and increases are approximately twice as much as with thrombin. The ADP-induced MLC phosphorylation is transient and reverses within 1 min[136,137]. However, during the first 5 s after stimulation MLC phosphorylation by ADP[138] or by thrombin[92] is not well correlated with Ca^{2+}-dynamics. This suggests that other mechanisms are also involved, possibly Rho-kinase. The early kinetics (<5 s) of other serine/threonine protein phosphorylations potentially associated with the SC are not known, nor for phosphotyrosine-containing proteins.

Effect of platelet inhibitors

A number of compounds regulate platelet protein kinases/and phosphatases and cause platelets to lose their disc shape and develop pseudopodia. Nevertheless, treatment with these agents appears to desensitize platelets to further stimulation with physiological agonists and block their physiological responses. Increases in cyclic AMP which inhibit platelet activation can actually induce pseudopod-like structures, indicating PKA activation of PKA may cause cytoskeletal reorganization leading to a platelet SC[139,140]. Inhibitors of serine/threonine protein phosphatases types 1 (PP1) and 2A (PP2A), okadaic acid (OA) and calyculin A (CLA), also induce changes in platelet morphology[132,141] that are correlated with inhibition of platelet aggregation and secretion. The phosphotyrosine phosphatase inhibitor vanadyl hydroperoxide induces morphological alterations in platelets that correlate with increased tyrosine phosphorylation and potentiation of cell adhesion. In ADP-stimulated platelets, the cGMP-elevating agent, sodium nitroprusside, inhibits SC by 50%[142].

Phosphoinositides and the SC

Polyphosphoinositides (PPI) play a key role in the reorganization of the actin filament network in different cells, including platelets by interacting with proteins that modulate actin assembly such as gelsolin and capping protein[18,99,143,144], profilin[144–147], cofilin[128,130,148–150] and the ERM protein, moesin[119]. Bursts of actin polymerization in vivo involve the transient appearance of free barbed-ends. It is thought that the two most abundant barbed-end capping proteins gelsolin and capping protein can be released from capped actin filament ends by interacting with the PIP_2 and PIP. In platelets, uncapping of gelsolin-capped filaments seems to generate free barbed ends and contributes to actin polymerization. Uncapping would occur at the membrane so that additional actin polymer-

Cambridge Books Online © Cambridge University Press, 2009

ization would result in formation of filopodia and lamellipodia. A recent review[151] summarizes the role of polyphosphoinositides in forming signalling protein complexes and regulating actin assembly in human platelets, focusing on pleckstrin homology (PH) domains. Platelet stimulation induces rapid phosphoinositide hydrolysis that is associated with actin polymerization and PIP_2 binding to actin-capping proteins, leading to the generation of actin-free ends and initiation of actin polymerization. Important interactions occur between the phosphoinositide kinases and small GTP-binding proteins such as Rac, Cdc42, and Rho, that are implicated in controlling the organization of actin cytoskeleton[152]. Thus, activation of Cdc42 may lead to rapid filopodial protrusions[152,153]. Activation of Rac causes fibroblasts to extend lamellipodia[154] while Rho is thought to be involved in the formation of stress fibres and focal contacts[155]. In platelets, Rac stimulates the uncapping of actin filaments through a PPI-dependent mechanism[156]. Rho and Rac regulate phosphatidylinositol 4-phosphate 5 kinase (PIP-5K) that is necessary for PIP_2 production[156–159]. Platelet stimulation with thrombin leads to activation of PI3–K and production of $PI(3,4)P_2$ and $PI(3,4,5)P_3$, that correlates with the kinetics of actin assembly and shape change[95]. The production of $PI(3,4)P_2$ correlates with the translocation of PI3–K to the platelet actin cytoskeleton and an increase in PI3–K enzyme activity[160]. Both activated-Rac and -Cdc42 associate with PI3–K. Interestingly, the inhibition of PI3–K activity by specific inhibitors such wortmannin does not alter the kinetics of actin assembly and shape change induced by TRAP or ADP suggesting that PI3–K is not required for actin assembly[93,161].

Shape change and disease: clinical relevance

There are relatively few examples of platelet-specific SC abnormalities associated with pathological situations, suggesting that an inability to undergo efficient SC may prove fatal in embryonic development. However, there are some conditions in humans and in animals such as dogs, where abnormal SC can occur. More common are clinical situations where an abnormal plasma environment influences platelet function. This section will consider known examples and also the usefulness of SC measurement in the important area of platelet storage in blood banks.

There is evidence that hypertension is associated with an enhanced SC measured optically[162–164]. Surface expression of CD62P (P-selectin) measured by flow cytometry is enhanced in acute and recent cerebrovascular ischemia, while the proportion of resting discoid platelets assessed by phase-contrast microscopy is unchanged compared to control subjects[55]. Thus, the information obtained and conclusions reached can be influenced by the techniques used. SC in unstable angina is enhanced three–fourfold compared to controls when assessed by the amplitude of the optical tracing[165], while exercise of postmyocardial infarction patients caused a significant activation of platelet morphology judged by light microscopy detection of the appearance of pseudopodia and loss of discoid shape[166].

Several other conditions or diseases are linked to SC abnormalities. Platelets in the rare **May-Hegglin anomaly** are large (mean volume of about 12 fl) and spherical and the disease is characterized by thrombocytopenia and mild bleeding[167]. Platelet aggregation induced by ADP or collagen is apparently normal, while SC in 15 subjects was completely absent as reflected in the optical tracings. Electron microscope studies indicate limited pseudopodia formation[168]. Abnormal SC has been reported in several other giant-platelet syndromes[169], including the Bernard–Soulier syndrome. Wilson's disease represents another situation where SC is usually absent[170], associated with a mild thrombocytopenia and a bruising tendency.

Platelet SC may be modulated abnormally by plasma factors in certain diseases. Thus, in both **thrombotic thrombocytopenic purpura** (TTP) and **hemolytic uremic syndrome** (HUS), inflammation and disturbances in endothelial cell function may contribute to the SC problem by releasing proteins and platelet-active substances into the blood[171]. An early indication of plasma factors which could induce the platelet SC was reported in1975[172]. Plasma from subjects with **acute** or **chronic heart disease** caused normal platelets to exhibit spread forms as visualized by electron microscopy. A number of factors might be responsible for these SC plasma effects, including oxidized-LDL[121,173], platelet-active amines such as epinephrine, or serotonin (5–OH tryptamine)[50,174], and most recently, chemokines which may link inflammatory conditions with cardiovascular disease and platelet activation[52,175–178].

An interesting linkage exists between the disease **hereditary gelsolin-related amyloidosis** (A Gal amyloidosis) and defective platelet SC induced by ADP or collagen[100]. This disorder is caused by a G654A or G654T mutation in the gene coding for gelsolin, an actin filament severing protein, as described earlier. Only light scattering was used to study SC and more direct information by SEM would have been valuable since gelsolin is believed to play a major role in the formation of filopodia and lamellipodia.

Measurement of platelet volume provides a useful index of the SC as outlined earlier. The existence of large platelets has been described for a variety of diseases or conditions[46]. Thus, large platelets may be associated with the development of atherosclerosis[179,180], although interpretations of

Cambridge Books Online © Cambridge University Press, 2009

such relationships are compounded by the fact that platelet populations are heterogeneous in size, density and other parameters[8,181–183]. For example, large platelets may be more sensitive to an aggregating agent than smaller cells, but their maximal rates of aggregation are lower[8,13,181]. Large, dense platelets adhere to collagen much more rapidly than lighter, small cells[184], causing the non-adherent platelet population to appear smaller. An interesting example of volume changes reflecting the platelet SC and relevant to clinical situations was recently reported. The drug Naftidrofuryl (NAF), which improves claudication distance associated with peripheral vascular disease (PVD), prevents the ability of the vasoconstrictor endothelin-1 and 5–OH tryptamine (serotonin) to cause the platelet SC sensed as an increase in mean platelet volume[174].

Several areas of research suggest the utility of SC measurements in **non-disease situations**. Thus, optical analysis of the ability of ADP to induce a SC in EDTA-treated platelets to minimize aggregation, and by a hypotonic-shock test indicate good correlation with in vivo platelet survival[185]. This information has proved important for developing optimal in vitro storage conditions of platelet concentrates. The effects of diet and environmental factors on platelet function, usually studied as light-scattering aggregation, have been studied for many years, often without clear resolution. The antioxidant Vitamin E does not block platelet aggregation, even at high dietary supplementation. However, much lower doses of Vitamin E it strongly inhibit adhesion under flow to surfaces coated with fibrinogen or collagen[186]. Examination of adherent platelets by SEM revealed that the SC of platelets from Vitamin E-supplemented individuals was much reduced, with few blunt pseudopodia compared to the longer narrower pseudopodia of platelets from control individuals. The reasons for dietary Vitamin E blocking the SC caused by platelet adhesion are not clear[186].

Conclusions and questions for the future

Conclusions

1. The shape change (SC) represents one of the earliest functional events (<5 s) after platelet stimulation. It is characterized by the loss of resting discoid shape with the formation of more rounded cells which initially exhibit short blebs and later, longer pseudopodia.
2. Detection and definition of the SC depend on the methods used for its study. Four main ones are most appropriate and combinations are recommended: (*a*) changes in the light-scattering properties of platelet suspensions; (*b*) changes in morphology assessed by light microscopy and by transmission, scanning and freeze-fracture electron microscope; (*c*) changes in platelet volume detected by resistive-particle counters; and, (*d*) by exposure of 'new' surface epitopes, by techniques such as flow cytometry.
3. Two major cell-signalling pathways mediate the SC, Ca^{2+}-dependent and independent ones, and simultaneous inhibition of both blocks SC. The former involves G_q-linked activation of the myosin light-chain kinase (MLCK), and the latter $G_{12/13}$ activation of Rho, leading to stimulation of Rho- kinase and inhibition of myosin phosphatase, also promoting phosphorylation of myosin light chains.
4. The important role of serine/threonine phosphorylation in regulating the SC is supported by the fact that okadaic acid, which inhibits protein phosphatase-1 and -2A (PP1 and PP2A) activities induces strong changes in platelet shape.
5. Evidence for roles of protein tyrosine phosphorylation is more limited. Src-kinase inhibitors do not prevent the ADP or thrombin-induced SC, while the collagen-related peptide (CRP) induced SC can be completely prevented. Tyrosine phosphorylation of cortactin, $p72^{Syk}$ and $p60^{src}$, and protein bands at 62, 68 and 130 kDa has been noted for various agonists, but direct relevance to the SC has yet to be established.
6. A number of additional proteins and factors are involved in the SC. Moesin, a linker protein between the plasma membrane and the actin cytoskeleton, is transiently phosphorylated and redistributed to the membrane skeleton and into pseudopodia. Other proteins include cofilin which is dephosphorylated during the SC, moving from the cytoplasm to the actin cytoskeleton, and adducin a membrane-skeleton protein associated with F-actin, and which can be phosphorylated.
7. Phosphoinositides play important roles in the SC and organization of the actin cytoskeleton, by interaction with proteins such as gelsolin and profilin, and by tethering proteins containing pleckstrin homology or 'PH' domains, to form signalling complexes.
8. Platelet-specific disorders of the SC are rare, while abnormal SC is associated with a number of clinical situations. Thus, cardiovascular disease such as hypertension, angina, ischemia or congestive heart failure, may be linked to an enhanced SC. In addition, inflammatory conditions such as TTP and HUS are associated with platelet activation, including the SC.

Cambridge Books Online © Cambridge University Press, 2009

Future avenues for research

A number of issues about the platelet SC need to be addressed to help our understanding of the fundamental mechanisms involved in initiating and regulating the SC and linkage to overall aspects of platelet function with correlations with clinical situations. The topics outlined below provide a window on where profitable research is likely.

1. The important question of whether the SC is essential for subsequent platelet function, particularly aggregation, should be resolved; or, is the SC more related to the role of platelets in the clotting cascades?
2. There is a critical need to discover signal-transduction events that are specific to the SC. Only limited information is available about protein tyrosine phosphorylation and little is known about the earliest kinetics of signalling changes before aggregation begins at about 1 s after agonist activation. Such studies should be related to later events such as full, stable aggregation.
3. Information is also needed about initial SC and concurrent signalling events occurring during platelet adhesion to surfaces such as collagen or fibrinogen under static and flowing conditions. This should be contrasted with knowledge about changes seen during solution activation as has been the situation for almost all studies to date.
4. The role of signalling complexes in maintaining resting platelet shape requires study, as well as how scaffold proteins and their partners, including associations with the plasma membrane, membrane skeleton and cytoskeleton may be involved.

References

1 Born GV. Observations on the change in shape of blood platelets brought about by adenosine diphosphate. *J Physiol (Lond)* 1970a; 209: 487–511.

2 Hovig T. Blood platelet surface and shape. A scanning electron microscopic study. *Scand J Haematol* 1970; 7: 420–427.

3 Mannucci PM, Sharp AA. Platelet volume and shape in relation to aggregation and adhesion. *Br J Haematol* 1967; 13: 604–617.

4 McLean JR, Veloso H. Change of shape without aggregation caused by ADP in rabbit platelets at low pH. *Life Sci* 1967; 6: 1983–1986.

5 Michal F, Born GV. Effect of the rapid shape change of platelets on the transmission and scattering of light through plasma. *Nat New Biol* 1971; 231: 220–222.

6 White JG, Clawson CC. Overview article: biostructure of blood platelets. *Ultrastruct Pathol* 1980a; 1: 533–558.

7 White JG, Clawson CC. The surface-connected canalicular system of blood platelets—a fenestrated membrane system. *Am J Pathol* 1980b; 101: 353–364.

8 Carty DJ, Gear AR. Fractionation of platelets according to size: functional and biochemical characteristics. *Am J Hematol* 1986; 21: 1–14.

9 Frojmovic MM, Milton JG. Physical, chemical and functional changes following platelet activation in normal and 'giant' platelets. *Blood Cells* 1983; 9: 359–382.

10 Holme S, Heaton A, Konchuba A, Hartman P. Light scatter and total protein signal distribution of platelets by flow cytometry as parameters of size. *J Lab Clin Med* 1988; 112: 223–231.

11 Oda A, Daley JF, Cabral C, Kang JH, Smith M, Salzman EW. Heterogeneity in filamentous actin content among individual human blood platelets. *Blood* 1992; 79: 920–927.

12 Paulus JM. Platelet size in man. *Blood* 1975; 46: 321–336.

13 Haver VM, Gear AR. Functional fractionation of platelets: aggregation kinetics and glycoprotein labeling of differing platelet population. *Thromb Haemost* 1982; 48: 211–216.

14 Opper C, Schrumpf E, Gear AR, Wesemann W. Involvement of guanylate cyclase and phosphodiesterases in the functional heterogeneity of human blood platelet subpopulations. *Thromb Res* 1995; 80: 461–470.

15 Opper C, Schuessler G, Kuschel M et al. Analysis of GTP-binding proteins, phosphoproteins, and cytosolic calcium in functional heterogeneous human blood platelet subpopulations. *Biochem Pharmacol* 1997; 54: 1027–1035.

16 Gear AR. Rapid platelet morphological changes visualized by scanning-electron microscopy: kinetics derived from a quenched-flow approach. *Br J Haematol* 1984; 56: 387–398.

17 Fox JE. The platelet cytoskeleton. *Thromb Haemost* 1993; 70: 884–893.

18 Hartwig JH. Mechanisms of actin rearrangements mediating platelet activation. *J Cell Biol* 1992; 118: 1421–1442.

19 Gear AR. Preaggregation reactions of platelets. *Blood* 1981; 58: 477–490.

20 Kitek A, Breddin K. Optical density variations and microscopic observations in the evaluation of platelet shape change and microaggregate formation. *Thromb Haemost* 1980; 44: 154–158.

21 Winokur R, Hartwig JH. Mechanism of shape change in chilled human platelets. *Blood* 1995; 85: 1796–1804.

22 Zucker MB, Grant RA. Nonreversible loss of platelet aggregability induced by calcium deprivation. *Blood* 1978; 52: 505–513.

23 George JN, Nurden AT. Inherited disorders of the platelet membrane: Glanzmann Thrombasthenia, Bernard–Soulier Syndrome, and other disorders. *Hemostasis and thrombosis; basic principles and clinical practice*. Philadelphia: JB Lippincott Company.

24 O'Brien JR. Effects of adenosine diphosphate and adrenaline on mean platelet shape. *Nature* 1965; 207: 306–307.

25 Gear AR, Lambrecht JK. Reduction in single platelets during primary and secondary aggregation [letter]. *Thromb Haemost* 1981; 45: 298.

Cambridge Books Online © Cambridge University Press, 2009

26 Latimer P, Born GV, Michal F. Application of light-scattering theory to the optical effects associated with the morphology of blood platelets. *Arch Biochem Biophys* 1977; 180: 151–159.
27 O'Brien JR, Heywood JB. Effect of aggregating agents and their inhibitors on the mean platelet shape. *J Clin Pathol* 1966; 19: 148–153.
28 Frojmovic MM. Rheooptical studies of platelet structure and function. *Prog Hemost Thromb* 1978; 4: 279–319.
29 Hantgan RR. A study of the kinetics of ADP-triggered platelet shape change. *Blood* 1984; 64: 896–906.
30 Born GV. Observations on the rapid morphological reaction of platelets to aggregating agents. *Ser Haematol* 1970b; 3: 114–120.
31 Bauer M, Retzer M, Wilde JI et al. Dichotomous regulation of myosin phosphorylation and shape change by Rho-kinase and calcium in intact human platelets. *Blood* 1999; 94: 1665–1672.
32 Wilde JI, Retzer M, Siess W, Watson SP. ADP-induced platelet shape change: an investigation of the signalling pathways involved and their dependence on the method of platelet preparation [In Process Citation]. *Platelets*, 2000; 11: 286–295.
33 White JG. Effects of ethylenediamine tetracetic acid (EDTA) on platelet structure. *Scand J Haematol* 1968a; 5: 241–254.
34 White JG. EDTA-induced changes in platelet structure and function: clot retraction. *Platelets*, 2000; 11: 49–55.
35 White JG, Escolar G. EDTA-induced changes in platelet structure and function: adhesion and spreading. *Platelets* 2000; 11: 56–61.
36 White JG. Fine structural alterations induced in platelets by adenosine diphosphate. *Blood* 1968b; 31: 604–622.
37 Gear AR. Platelet adhesion, shape change, and aggregation: rapid initiation and signal transduction events. *Can J Physiol Pharmacol* 1994; 72: 285–294.
38 Geanacopoulos M, Gear AR. Application of spray-freezing to the study of rapid platelet reactions by a quenched-flow approach. *Thromb Res* 1988; 52: 599–607.
39 Bull BS, Zucker MB. Changes in platelet volume produced by temperature, metabolic inhibitors, and aggregating agents. *Proc Soc Exp Biol Med* 1965; 120: 296–301.
40 Salzman EW, Ashford TP, Chambers DA, Neri LL, Dempster AP. Platelet volume: effect of temperature and agents affecting platelet aggregation. *Am J Physiol* 1969; 217: 1330–1338.
41 Zucker MB, Borrelli J. Reversible alterations in platelet morphology produced by anticoagulants and by cold. *Blood* 1954; 9: 602–608.
42 Feinberg H, Sandler WC, Scorer M, Le Breton GC, Grossman B, Born GV. Movement of sodium into human platelets induced by ADP. *Biochim Biophys Acta* 1977; 470: 317–324.
43 Le Breton GC, Dinerstein RJ, Roth LJ, Feinberg H. Direct evidence for intracellular divalent cation redistribution associated with platelet shape change. *Biochem Biophys Res Commun* 1976; 71: 362–370.
44 Sandler WC, Le Breton GC, Feinberg H. Movement of sodium into human platelets. *Biochim Biophys Acta* 1980; 600: 448–455.
45 Milton JG, Frojmovic MM. Turbidometric evaluations of platelet activation: relative contributions of measured shape change, volume, and early aggregation. *J Pharmacol Methods* 1983; 9: 101–115.
46 Jackson SR, Carter JM. Platelet volume: laboratory measurement and clinical application. *Blood Rev* 1993; 7: 104–113.
47 Jagroop IA, Clatworthy I, Lewin J, Mikhailidis DP. Shape change in human platelets: measurement with a channelyzer and visualisation by electron microscopy. *Platelets* 2000; 11: 28–32.
48 Gear AR. Rapid reactions of platelets studied by a quenched-flow approach: aggregation kinetics. *J Lab Clin Med* 1982; 100: 866–886.
49 Born GV, Richardson PD. Activation time of blood platelets. *J Membr Biol* 1980; 57: 87–90.
50 Steen VM, Holmsen H, Aarbakke G. The platelet-stimulating effect of adrenaline through alpha 2-adrenergic receptors requires simultaneous activation by a true stimulatory platelet agonist. Evidence that adrenaline per se does not induce human platelet activation in vitro. *Thromb Haemost* 1993; 70: 506–513.
51 Lalau Keraly C, Kinlough-Rathbone RL, Packham MA, Suzuki H, Mustard JF. Conditions affecting the responses of human platelets to epinephrine. *Thromb Haemost* 1988; 60: 209–216.
52 Gear ARL, Suttitanamongkol D, Viisoreanu D, Polanowska-Grabowska RK, Raha S, Camerini D. Adenosine diphosphate strongly potentiates the ability of the chemokines MDC, TARC, and SDF-1 to stimulate platelet function. *Blood* 2001; 97: 937–945.
53 Shattil SJ, Cunningham M, Hoxie JA. Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. *Blood* 1987; 70: 307–315.
54 Schmitz G, Rothe G, Ruf A et al. European Working Group on Clinical Cell Analysis: Consensus protocol for the flow cytometric characterisation of platelet function. *Thromb Haemost* 1998; 79: 885–896.
55 Grau AJ, Ruf A, Vogt A et al. Increased fraction of circulating activated platelets in acute and previous cerebrovascular ischemia. *Thromb Haemost* 1998; 80: 298–301.
56 Gallet C, Rosa JP, Habib A, Lebret M, Levy-Toledano S, Maclouf J. Tyrosine phosphorylation of cortactin associated with Syk accompanies thromboxane analogue-induced platelet shape change. *J Biol Chem* 1999; 274: 23610–23616.
57 Negrescu EV, de Quintana KL, Siess W. Platelet shape change induced by thrombin receptor activation. Rapid stimulation of tyrosine phosphorylation of novel protein substrates through an integrin- and $Ca^{(2+)}$-independent mechanism. *J Biol Chem* 1995a; 270: 1057–1061.
58 Daniel JL, Molish IR, Rigmaiden M, Stewart G. Evidence for a role of myosin phosphorylation in the initiation of the platelet shape change response. *J Biol Chem* 1984; 259: 9826–9831.
59 Rink TJ, Smith SW, Tsien RY. Cytoplasmic free Ca^{2+} in human platelets: Ca^{2+} thresholds and Ca-independent activation for shape-change and secretion. *FEBS Lett* 1982; 148: 21–26.

Cambridge Books Online © Cambridge University Press, 2009

60 Jones GD, Gear AR. Subsecond calcium dynamics in ADP- and thrombin-stimulated platelets: a continuous-flow approach using indo-1. *Blood* 1989; 71: 1539–1543.
61 Sage SO, Rink TJ. The kinetics of changes in intracellular calcium concentration in fura-2-loaded human platelets. *J Biol Chem* 1987; 262: 16364–16369.
62 Ohkubo S, Nakahata N, Ohizumi Y. Thromboxane A2-mediated shape change: independent of Gq-phospholipase C–Ca^{2+} pathway in rabbit platelets. *Br J Pharmacol* 1996; 117: 1095–1104.
63 Simpson AW, Hallam TJ, Rink TJ. Low concentrations of the stable prostaglandin endoperoxide U44069 stimulate shape change in quin2-loaded platelets without a measurable increase in $[Ca^{2+}]_i$. *FEBS Lett* 1986; 201: 301–305.
64 Rasmussen UB, Gachet C, Schlesinger Y et al. A peptide ligand of the human thrombin receptor antagonizes alpha-thrombin and partially activates platelets. *J Biol Chem* 1993; 268: 14322–14328.
65 Siess W, Zangl KJ, Essler M et al. Lysophosphatidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions. *Proc Natl Acad Sci USA* 1999; 96: 6931–6936.
66 Offermanns S, Toombs CF, Hu YH, Simon MI. Defective platelet activation in G alpha(q)-deficient mice. *Nature* 1997; 389: 183–186.
67 Paul BZ, Daniel JL, Kunapuli SP. Platelet shape change is mediated by both calcium-dependent and -independent signaling pathways. Role of p160 Rho-associated coiled-coil-containing protein kinase in platelet shape change. *J Biol Chem* 1999; 274: 28293–28300.
68 Nachmias VT, Kavaler J, Jacubowitz S. Reversible association of myosin with the platelet cytoskeleton. *Nature* 1985; 313: 70–72.
69 Fox JE, Phillips DR. Role of phosphorylation in mediating the association of myosin with the cytoskeletal structures of human platelets. *J Biol Chem* 1982; 257: 4120–4126.
70 de Chaffoy de Courcelles D, Roevens P, van Belle H. Stimulation by serotonin of 40 kDa and 20 kDa protein phosphorylation in human platelets. *FEBS Lett* 1984; 171: 289–292.
71 Siess W, Boehlig B, Weber PC, Lapetina EG. Prostaglandin endoperoxide analogues stimulate phospholipase C and protein phosphorylation during platelet shape change. *Blood* 1985; 65: 1141–1148.
72 Siess W, Lapetina EG. Ca^{2+} mobilization primes protein kinase C in human platelets. Ca^{2+} and phorbol esters stimulate platelet aggregation and secretion synergistically through protein kinase C. *Biochem J* 1988; 255: 309–318.
73 Lanza F, Beretz A, Stierle A, Hanau D, Kubina M, Cazenave JP. Epinephrine potentiates human platelet activation but is not an aggregating agent. *Am J Physiol* 1988; 255; H1276–H1288.
74 Siess W. Evidence for the formation of inositol 4-monophosphate in stimulated human platelets. *FEBS Lett* 1985; 185: 151–156.
75 Siess W, Binder H. Thrombin induces the rapid formation of inositol bisphosphate and inositol trisphosphate in human platelets. *FEBS Lett* 1985; 180: 107–112.
76 Somlyo AP, Somlyo AV. Signal transduction and regulation in smooth muscle [published erratum appears in *Nature* 1994 Dec 22–29: 372(6508): 812]. *Nature* 1994; 372: 231–236.
77 Nakai K, Suzuki Y, Kihira H et al. Regulation of myosin phosphatase through phosphorylation of the myosin-binding subunit in platelet activation. *Blood* 1997; 90: 3936–3942.
78 Suzuki Y, Yamamoto M, Wada et al. Agonist-induced regulation of myosin phosphatase activity in human platelets through activation of Rho-kinase. *Blood* 1999; 93: 3408–3417.
79 Carty DJ, Freas DL, Gear AR. ADP causes subsecond changes in protein phosphorylation of platelets. *Blood* 1987; 70: 511–515.
80 Paul BZ, Jin J, Kunapuli SP. Molecular mechanism of thromboxane A(2)-induced platelet aggregation. Essential role for p2t(ac) and alpha(2a) receptors. *J Biol Chem* 1999; 274: 29108–29114.
81 Klages B, Brandt U, Simon MI, Schultz G, Offermanns S. Activation of G12/G13 results in shape change and Rho/Rho-kinase-mediated myosin light chain phosphorylation in mouse platelets. *J Cell Biol* 1999; 144: 745–754.
82 Fabre JE, Nguyen M, Latour A et al. Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y1-deficient mice. *Nat Med* 1999; 5: 1199–1202.
83 Ohlmann P, Eckly A, Freund M, Cazenave JP, Offermanns S, Gachet C. ADP induces partial platelet aggregation without shape change and potentiates collagen-induced aggregation in the absence of Galphaq. *Blood* 2000; 96: 2134–2139.
84 Brazell C, McClue SJ, Preston GC, King B, Stahl SM. 5-Hydroxytryptamine (5-HT)-induced shape change in human platelets determined by computerized data acquisition: correlation with [125I]-iodoLSD binding at 5-HT2 receptors. *Blood Coagul Fibrinol* 1991; 2: 17–24.
85 Jin J, Daniel JL, Kunapuli SP. Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J Biol Chem* 1998; 273: 2030–2034.
86 Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. *Proc Natl Acad Sci USA* 1998; 95: 8070–8074.
87 Rosa JP, Artcanuthurry V, Grelac F, Maclouf J, Caen JP, Levy-Toledano S. Reassessment of protein tyrosine phosphorylation in thrombasthenic platelets: evidence that phosphorylation of cortactin and a 64-kD protein is dependent on thrombin activation and integrin alphaIIb beta3. *Blood* 1997; 89: 4385–4392.
88 Negrescu EV, Grunberg B, Kratzer MA, Lorenz R, Siess W. Interaction of antiplatelet drugs in vitro: aspirin, iloprost, and the nitric oxide donors SIN-1 and sodium nitroprusside. *Cardiovasc Drugs Ther* 1995b; 9: 619–629.
89 Briddon SJ, Melford SK, Turner M, Tybulewicz V, Watson SP. Collagen mediates changes in intracellular calcium in primary mouse megakaryocytes through syk-dependent and -independent pathways. *Blood* 1999; 93: 3847–3855.

Cambridge Books Online © Cambridge University Press, 2009

90 Maschberger P, Bauer M, Baumann-Siemons J et al. Mildly oxidized low density lipoprotein rapidly stimulates via activation of the lysophosphatidic acid receptor Src family and Syk tyrosine kinases and Ca^{2+} influx in human platelets. *J Biol Chem* 2000; 275: 19159–19166.
91 Carty DJ, Spielberg F, Gear AR. Thrombin causes subsecond changes in protein phosphorylation of platelets. *Blood* 1986; 67: 1738–1743.
92 Jones GD, Carty DJ, Freas DL, Spears JT, Gear AR. Effects of separate proteolytic and high-affinity binding activities of human thrombin on rapid platelet activation. A quenched-flow study. *Biochem J* 1989; 262: 611–616.
93 Hashimoto Y, Sasaki H, Togo M et al. Roles of mysosin light-chain kinase in platelet shape change and aggregation *Biochim Biophys Acta* 1994; 1223: 163–169.
94 Puri RN, Colman RW. Thrombin- and cathepsin G-induced platelet aggregation: effect of protein kinase C inhibitors. *Anal Biochem* 1993; 210: 50–57.
95 Hartwig JH, Kung S, Kovacsovics T et al. D3 phosphoinositides and outside-in integrin signaling by glycoprotein IIb–IIIa mediate platelet actin assembly and filopodial extension induced by phorbol 12-myristate 13-acetate. *J Biol Chem* 1996; 271: 32986–32993.
96 Hartwig JH, DeSisto M. The cytoskeleton of the resting human blood platelet: structure of the membrane skeleton and its attachment to actin filaments. *J Cell Biol* 1991; 112: 407–425.
97 Siess W. Molecular mechanisms of platelet activation. *Physiol Rev* 1989; 69: 58–178.
98 Wurzinger LJ. Histophysiology of the circulating platelet. *Adv Anat Embryol Cell Biol* 1990; 120: 1–96.
99 Hartwig JH. Platelet morphology. In Loscalzo JL, Schafer AI (eds) *Thrombosis and hemorrhage*. Baltimore, Philadelphia, London, Paris, Bangkok: Williams & Wilkins; 1998: 207–228.
100 Witke W, Sharpe AH, Hartwig JH, Azuma T, Stossel TP, Kwiatkowski DJ. Hemostatic, inflammatory, and fibroblast responses are blunted in mice lacking gelsolin. *Cell* 1995; 81: 41–51.
101 Kiuru S, Javela K, Somer H, Kekomaki R. Altered platelet shape change in hereditary gelsolin Asp187Asn-related amyloidosis. *Thromb Haemost* 2000; 83: 491–495.
102 Halbrugge M, Friedrich C, Eigenthaler M, Schanzenbacher P, Walter U. Stoichiometric and reversible phosphorylation of a 46-kDa protein in human platelets in response to cGMP- and cAMP-elevating vasodilators. *J Biol Chem* 1990; 265: 3088–3093.
103 Reinhard M, Halbrugge M, Scheer U, Wiegand C, Jockusch BM, Walter U. The 46/50 kDa phosphoprotein VASP purified from human platelets is a novel protein associated with actin filaments and focal contacts. *Embo J* 1992; 11: 2063–2070.
104 Walter U, Eigenthaler M, Geiger J, Reinhard M. Role of cyclic nucleotide-dependent protein kinases and their common substrate VASP in the regulation of human platelets. *Adv Exp Med Biol* 1993; 344: 237–249.
105 Bearer EL, Prakash JM, Manchester RD, Allen PG. VASP protects actin filaments from gelsolin: an in vitro study with implications for platelet actin reorganizations [In Process Citation]. *Cell Motil Cytoskeleton* 2000; 47: 351–364.
106 Harbeck B, Huttelmaier S, Schluter K, Jockusch BM, Illenberger S. Phosphorylation of the vasodilator-stimulated phosphoprotein regulates its interaction with actin. *J Biol Chem* 2000; 275: 30817–30825.
107 Aszodi A, Pfeifer A, Ahmad M et al. The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function. *Embo J* 1999; 18: 37–48.
108 Hauser W, Knobeloch KP, Eigenthaler M et al. Megakaryocyte hyperplasia and enhanced agonist-induced platelet activation in vasodilator-stimulated phosphoprotein knockout mice. *Proc Natl Acad Sci USA* 1999; 96: 8120–8125.
109 Polanowska-Grabowska R, Gear AR. Heat-shock proteins and platelet function. *Platelets* 2000; 11: 6–22.
110 Zhu Y, O'Neill S, Saklatvala J, Tassi L, Mendelsohn ME. Phosphorylated HSP27 associates with the activation-dependent cytoskeleton in human platelets. *Blood* 1994; 84: 3715–3723.
111 Landry J, Huot J. Regulation of actin dynamics by stress-activated protein kinase 2 (SAPK2)-dependent phosphorylation of heat-shock protein of 27 kDa (Hsp27). *Biochem Soc Symp* 1999; 64: 79–89.
112 Lavoie JN, Gingras-Breton G, Tanguay RM, Landry J. Induction of Chinese hamster HSP27 gene expression in mouse cells confers resistance to heat shock. HSP27 stabilization of the microfilament organization. *J Biol Chem* 1993; 268: 3420–3429.
113 Schneider GB, Hamano H, Cooper LF. In vivo evaluation of hsp27 as an inhibitor of actin polymerization: hsp27 limits actin stress fiber and focal adhesion formation after heat shock. *J Cell Physiol* 1998; 177: 575–584.
114 Schafer C, Clapp P, Welsh MJ, Benndorf R, Williams JA. HSP27 expression regulates CCK-induced changes of the actin cytoskeleton in CHO-CCK-A cells. *Am J Physiol* 1999; 277; C1032–C1043.
115 Butt E, Immler D, Meyer HE, Kotlyarov A, Laass K, Gaestel M. Heat shock protein 27 is a substrate of cGMP-dependent protein kinase in intact human platelets. Phosphorylation-induced actin polymerization caused by Hsp27 mutants. *J Biol Chem* 2000; 30: 30.
116 Polanowska-Grabowska R, Simon CG, Jr., Falchetto R, Shabanowitz J, Hunt DF, Gear AR. Platelet adhesion to collagen under flow causes dissociation of a phosphoprotein complex of heat-shock proteins and protein phosphatase 1. *Blood* 1997; 90: 1516–1526.
117 Tsukita S, Yonemura S. ERM (ezrin/radixin/moesin) family: from cytoskeleton to signal transduction. *Curr Opin Cell Biol* 1997; 9: 70–75.
118 Nakamura F, Amieva MR, Furthmayr H. Phosphorylation of threonine 558 in the carboxyl-terminal actin-binding domain of moesin by thrombin activation of human platelets. *J Biol Chem* 1995; 270: 31377–31385.

Cambridge Books Online © Cambridge University Press, 2009

119 Nakamura F, Huang L, Pestonjamasp K, Luna EJ, Furthmayr H. Regulation of F-actin binding to platelet moesin in vitro by both phosphorylation of threonine 558 and polyphosphatidylinositides. *Mol Biol Cell* 1999; 10: 2669–2685.
120 Shcherbina A, Kenney DM, Bretscher A, Remold ODE. Dynamic association of moesin with the membrane skeleton of thrombin-activated platelets. *Blood* 1999; 93: 2128–2129.
121 Mackay DJ, Esch F, Furthmayr H, Hall A. Rho- and rac-dependent assembly of focal adhesion complexes and actin filaments in permeabilized fibroblasts: an essential role for ezrin/radixin/moesin proteins. *J Cell Biol* 1997; 138: 927–938.
122 Retzer M, Siess W, Essler M. Mildly oxidised low density lipoprotein induces platelet shape change via Rho-kinase-dependent phosphorylation of myosin light chain and moesin. *FEBS Lett* 2000; 466: 70–74.
123 Doi Y, Itoh M, Yonemura S et al. Normal development of mice and unimpaired cell adhesion/cell motility/actin-based cytoskeleton without compensatory up-regulation of ezrin or radixin in moesin gene knockout. *J Biol Chem* 1999; 274: 2315–2321.
124 Gardner K, Bennett V. Modulation of spectrin-actin assembly by erythrocyte adducin. *Nature* 1987; 328: 359–362.
125 Fukata Y, Oshiro N, Kinoshita N et al. Phosphorylation of adducin by Rho-kinase plays a crucial role in cell motility. *J Cell Biol* 1999; 145: 347–361.
126 Kimura K, Fukata Y, Matsuoka Y et al. Regulation of the association of adducin with actin filaments by Rho-associated kinase (Rho-kinase) and myosin phosphatase. *J Biol Chem* 1998; 273: 5542–5548.
127 Matsuoka Y, Hughes CA, Bennett V. Adducin regulation. Definition of the calmodulin-binding domain and sites of phosphorylation by protein kinases A and C. *J Biol Chem* 1996; 271: 25157–25166.
128 Matsuoka Y, Li X, Bennett V. Adducin is an in vivo substrate for protein kinase C: phosphorylation in the MARCKS-related domain inhibits activity in promoting spectrin-actin complexes and occurs in many cells, including dendritic spines of neurons. *J Cell Biol* 1998; 142: 485–497.
129 Yonezawa N, Nishida E, Sakai H. pH control of actin polymerization by cofilin. *J Biol Chem* 1985; 260: 14410–14412.
130 Davidson MM, Haslam RJ. Dephosphorylation of cofilin in stimulated platelets: roles for a GTP-binding protein and Ca^{2+}. *Biochem J* 1994; 301: 41–47.
131 Yonezawa N, Nishida E, Iida K, Yahara I, Sakai H. Inhibition of the interactions of cofilin, destrin, and deoxyribonuclease I with actin by phosphoinositides. *J Biol Chem* 1990; 265: 8382–8386.
132 Kurisaki T, Taylor RG, Hartshorne DJ. Effects of the protein phosphatase inhibitors, tautomycin and calyculin-A, on protein phosphorylation and cytoskeleton of human platelets. *Cell Struct Funct* 1995; 20: 331–343.
133 Yano Y, Kambayashi J, Shiba E et al. The role of protein phosphorylation and cytoskeletal reorganization in microparticle formation from the platelet plasma membrane. *Biochem J* 1994; 299: 303–308.
134 Okada K, Takano-Ohmuro H, Obinata T, Abe H. Dephosphorylation of cofilin in polymorphonuclear leukocytes derived from peripheral blood. *Exp Cell Res* 1996; 227: 116–122.
135 Samstag Y, Dreizler EM, Ambach A, Sczakiel G, Meuer SC. Inhibition of constitutive serine phosphatase activity in T lymphoma cells results in phosphorylation of pp19/cofilin and induces apoptosis. *J Immunol* 1996; 156: 4167–4173.
136 Haslam RJ, Lynham JA. Relationship between phosphorylation of blood platelet proteins and secretion of platelet granule constituents. I. Effects of different aggregating agents. *Biochem Biophys Res Commun* 1977; 77: 714–722.
137 Haslam RJ, Lynham JA. Relationship between phosphorylation of blood platelet proteins and secretion of platelet granule constituents II. Effects of different inhibitors. *Thromb Res* 1978; 12: 619–628.
138 Carty DJ, Jones GD, Freas DL, Gear AR. Effect of epinephrine on rapid ADP-induced aggregation, protein phosphorylation, and cytoplasmic calcium dynamics of platelets: a quenched-flow study. *J Lab Clin Med* 1988; 112: 603–611.
139 Maurer DR, Majercik MH, Bourguignon LY. Effect of cyclic AMP on human blood platelets: induction of pseudopod formation and protein phosphorylation. *Cell Biol Int Rep* 1988; 12: 271–288.
140 Zilla P, Groscurth P, Varga G, Fischlein T, Fasol R. PGI2 and PGE1 induce morphological alterations in human platelets similar to those of the initial phase of activation. *Exp Hematol* 1987; 15: 741–749.
141 Yano Y, Sakon M, Kambayashi J et al. Cytoskeletal reorganization of human platelets induced by the protein phosphatase 1/2 A inhibitors okadaic acid and calyculin A. *Biochem J* 1995; 307: 439–449.
142 Morgan RO, Newby AC. Nitroprusside differentially inhibits ADP-stimulated calcium influx and mobilization in human platelets. *Biochem J* 1989; 258: 447–454.
143 Barkalow K, Witke W, Kwiatkowski DJ, Hartwig JH. Coordinated regulation of platelet actin filament barbed ends by gelsolin and capping protein. *J Cell Biol* 1996; 134: 389–399.
144 Lind SE, Janmey PA, Chaponnier C, Herbert TJ, Stossel TP. Reversible binding of actin to gelsolin and profilin in human platelet extracts. *J Cell Biol* 1987; 105: 833–842.
145 Goldschmidt-Clermont PJ, Machesky LM, Baldassare JJ, Pollard TD. The actin-binding protein profilin binds to PIP2 and inhibits its hydrolysis by phospholipase C. *Science* 1990; 247: 1575–1578.
146 Goldschmidt-Clermont PJ, Kim JW, Machesky LM, Rhee SG, Pollard TD. Regulation of phospholipase C-gamma 1 by profilin and tyrosine phosphorylation. *Science* 1991; 251: 1231–1233.
147 Lassing I, Lindberg U. Specificity of the interaction between phosphatidylinositol 4,5-bisphosphate and the profilin: actin complex. *J Cell Biochem* 1988; 37: 255–267.
148 Yahara I, Aizawa H, Moriyama K et al. A role of cofilin/destrin in reorganization of actin cytoskeleton in response to stresses and cell stimuli. *Cell Struct Funct* 1996; 21: 421–424.

Cambridge Books Online © Cambridge University Press, 2009

149 Yonezawa N, Homma Y, Yahara I, Sakai H, Nishida E. A short sequence responsible for both phosphoinositide binding and actin binding activities of cofilin. *J Biol Chem* 1991; 266: 17218–17221.
150 Yonezawa N, Nishida E, Iida K, Kumagai H, Yahara I, Sakai H. Inhibition of actin polymerization by a synthetic dodecapeptide patterned on the sequence around the actin-binding site of cofilin. *J Biol Chem* 1991; 266: 10485–10489.
151 Hartwig JH, Barkalow K. Polyphosphoinositide synthesis and platelet shape change. *Curr Opin Hematol* 1997; 4: 351–356.
152 Kjoller L, Hall A. Signaling to Rho GTPases. *Exp Cell Res* 1999; 253: 166–179.
153 Nobes CD, Hall A. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 1995; 81: 53–62.
154 Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* 1992; 70: 401–410.
155 Ridley AJ, Hall A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 1992; 70: 389–399.
156 Hartwig JH, Bokoch GM, Carpenter CL et al. Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. *Cell* 1995; 82: 643–653.
157 Azim AC, Barkalow K, Chou J, Hartwig JH. Activation of the small GTPases, rac and cdc42, after ligation of the platelet PAR-1 receptor. *Blood* 2000; 95: 959–964.
158 Chong LD, Traynor-Kaplan A, Bokoch GM, Schwartz MA. The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell* 1994; 79: 507–513.
159 Tolias KF, Hartwig JH, Ishihara H, Shibasaki Y, Cantley LC, Carpenter CL. Type Ialpha phosphatidylinositol-4-phosphate 5-kinase mediates Rac-dependent actin assembly. *Curr Biol* 2000; 10: 153–156.
160 Guinebault C, Payrastre B, Racaud-Sultan C et al. Integrin-dependent translocation of phosphoinositide 3-kinase to the cytoskeleton of thrombin-activated platelets involves specific interactions of p85 alpha with actin filaments and focal adhesion kinase. *J Cell Biol* 1995; 129: 831–842.
161 Kovacsovics TJ, Bachelot C, Toker A et al. Phosphoinositide 3-kinase inhibition spares actin assembly in activating platelets but reverses platelet aggregation. *J Biol Chem* 1995; 270: 11358–11366.
162 Amstein R, Fetkovska N, Luscher TF, Kiowski W, Buhler FR. Age and the platelet serotonin vasoconstrictor axis in essential hypertension. *J Cardiovasc Pharmacol* 1988; 11; S35–S40.
163 Erne P, Resink TJ, Bolli P, Hefti A, Ritz R, Buhler FR. Free calcium response to adrenaline in platelets of normal and hypertensive (untreated and treated) subjects. *J Hypertens Suppl* 1984; 2; S159–S161.
164 Lande K, Os I, Kjeldsen SE et al. Platelet volume, platelet release reaction and platelet response to infused adrenaline are increased in essential hypertension. *Acta Med Scand Suppl* 1986; 714: 129–132.
165 Zahavi M, Zahavi J, Schafer R, Firsteter E, Laniado S. Abnormal typical pattern of platelet function and thromboxane generation in unstable angina. *Thromb Haemost* 1989; 62: 840–845.
166 Douste-Blazy P, Sie P, Boneu B, Marco J, Eche N, Bernadet P. Exercise-induced platelet activation in myocardial infarction survivors with normal coronary arteriogram. *Thromb Haemost* 1984; 52: 297–300.
167 Noris P, Spedini P, Belletti S, Magrini U, Balduini CL. Thrombocytopenia, giant platelets, and leukocyte inclusion bodies (May–Hegglin anomaly): clinical and laboratory findings. *Am J Med* 1998; 104: 355–360.
168 Lusher JM, Barnhart MI. Congenital disorders affecting platelets. *Semin Thromb Haemost* 1977; 4: 123–186.
169 Milton JG, Hutton RA, Tuddenham, EG, Frojmovic, MM. Platelet size and shape in hereditary giant platelet syndromes on blood smear and in suspension: evidence for two types of abnormalities. *J Lab Clin Med* 1985; 106: 326–335.
170 Owen Jr CA, Goldstein NP, Bowie JW. Platelet function and coagulation in patients with Wilson disease. *Arch Intern Med* 1976; 136: 148–152.
171 Moake JL. Thrombotic microangiopathies: Thrombotic thrombocytpenic purpura and hemolytic uremic syndrome. Loscalzo, JLS, ed. *Thrombosis and hemorrhage*, Baltimore, Philadelphia, London, Paris, Bangkok: Williams & Wilkins; 1998: 583–598.
172 Schatz IJ, Gross I. Blood platelet response to plasma from patients with ischemic heart disease. *Am J Cardiol* 1975; 35: 204–210.
173 Weidtmann A, Scheithe R, Hrboticky N, Pietsch A, Lorenz R, Siess W. Mildly oxidized LDL induces platelet aggregation through activation of phospholipase A2. *Arterioscler Thromb Vasc Biol* 1995; 15: 1131–1138.
174 Jagroop IA, Mikhailidis DP. Effect of endothelin-1 on human platelet shape change: reversal of activation by naftidrofuryl [In Process Citation]. *Platelets* 2000; 11: 272–277.
175 Abi-Younes S, Sauty A, Mach F, Sukhova GK, Libby P, Luster AD. The stromal cell-derived factor-1 chemokine is a potent platelet agonist highly expressed in atherosclerotic plaques. *Circ Res* 2000; 86: 131–138.
176 Aukrust P, Ueland T, Muller F et al. Elevated circulating levels of C–C chemokines in patients with congestive heart failure. *Circulation* 1998; 97: 1136–1143.
177 Clemetson KJ, Clemetson JM, Proudfoot AE, Power CA, Baggiolini M, Wells TN. Functional expression of CCR1, CCR3, CCR4, and CXCR4 chemokine receptors on human platelets. *Blood* 2000; 96: 4046–4054.
178 Kowalska MA, Ratajczak MZ, Majka M et al. Stromal cell-derived factor-1 and macrophage-derived chemokine: 2 chemokines that activate platelets. *Blood* 2000; 96: 50–57.
179 Martin JF, Bath PM, Burr ML. Influence of platelet size on outcome after myocardial infarction. *Lancet* 1991; 338: 1409–1411.

Cambridge Books Online © Cambridge University Press, 2009

180 Trowbridge EA, Martin JF. The platelet volume distribution: a signature of the prethrombotic state in coronary heart disease? *Thromb Haemost* 1987; 58: 714–717.
181 Haver VM, Gear AR. Functional fractionation of platelets. *J Lab Clin Med* 1981; 97: 187–204.
182 Thompson CB, Jakubowski JA. The pathophysiology and clinical relevance of platelet heterogeneity. *Blood* 1988; 72: 1–8.
183 Thompson CB, Jakubowski JA, Quinn PG, Deykin D, Valeri CR. Platelet size as a determinant of platelet function. *J Lab Clin Med* 1983; 101: 205–213.
184 Polanowska-Grabowska R, Raha S, Gear AR. Adhesion efficiency, platelet density and size. *Br J Haematol* 1992; 82: 715–720.
185 Holme S, Moroff G, Murphy S. A multi-laboratory evaluation of in vitro platelet assays: the tests for extent of shape change and response to hypotonic shock. Biomedical Excellence for Safer Transfusion Working Party of the International Society of Blood Transfusion. *Transfusion* 1998; 38: 31–40.
186 Steiner M. Influence of vitamin E on platelet function in humans. *J Am Coll Nutr* 1991; 10: 466–473.
187 Bachmann L, Schmitt WW. Improved cryofixation applicable to freeze etching. *Proc Natl Acad Sci USA* 1971; 68: 2149–2152.

Cambridge Books Online © Cambridge University Press, 2009

23

Aggregation

Marian A. Packham[1], Margaret L. Rand[2] and Raelene L. Kinlough-Rathbone[3]

[1]*Department of Biochemistry, University of Toronto, Ontario, Canada*
[2]*Division of Hematology/Oncology, The Hospital for Sick Children, Toronto, Ontario, Canada*
[3]*Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada*

Introduction

Platelet aggregation is involved in the formation of hemostatic plugs and arterial thrombi. Under normal circumstances, platelets are non-adhesive and circulate singly, but following vessel wall injury they adhere to the injury site and to each other. During the hemostatic process, platelet aggregates, stabilized by fibrin, arrest bleeding from injured or severed vessels. In contrast to this useful function, platelet aggregates that form on injured vessels, on ruptured atherosclerotic plaques, or in regions of high shear contribute to the narrowing of blood vessels. If thrombi are unstable, they may embolize and block smaller vessels downstream from an injury site. Since platelet aggregation has a major role in the clinical complications of atherosclerosis (myocardial infarction, ischemic stroke, and peripheral vascular disease), there is intensive study of the processes involved in platelet aggregation and of inhibitors of platelet activation.

In vivo, activators of platelets include the agonists that are listed in Table 23.1. Receptors for some of the most important agonists are discussed in Chapters 8–11. Aggregating agents can act singly, and are frequently studied singly in vitro, but in vivo they undoubtedly act in concert with each other in a process described as synergism. Synergistic responses result in a combined effect that is greater than the additive effects of the single stimuli[1,2]. In vivo, the most important aggregating agents are collagen in the vessel wall, ADP from red blood cells or released from the platelets themselves, thromboxane A_2 formed by stimulated platelets, and thrombin, although other agonists such as serotonin may contribute to the aggregation process. Epinephrine alone does not cause platelet aggregation, but it can strongly enhance aggregation by other agonists[3–5].

Table 23.1. In vivo aggregating agents and conditions

ADP
Collagen
Arachidonic acid and prostaglandin endoperoxides PGG_2 and PGH_2, and thromboxane A_2
Thrombin and the cleaved peptide of the protease-activated receptor, PAR1
Epinephrine and norepinephrine[a]
Platelet activating factor (PAF)
Serotonin
Arginine vasopressin (AVP)
Thrombospondin
Cathepsin G
Plasmin
Bacteria and endotoxin, viruses, and parasites
Tumour cells
Immune complexes and antiplatelet antibodies
High levels of fluid shear stress

Note:
[a] Epinephrine is not a true aggregating agent, but exerts strong synergistic effects with other agonists (see text).

When an aggregating agent interacts with its receptor(s) on the platelet surface, intracellular signalling reactions occur that result in a change in the conformation of glycoprotein (GP)IIb/IIIa (αIIbβ3) (inside-out signalling) so that it becomes a receptor for fibrinogen or von Willebrand factor. Each of these proteins can form bridges between the stimulated platelets, leading to the formation of a mass of aggregated platelets[6]. Binding of fibrinogen or von Willebrand factor causes further changes in the conformation of GPIIb/IIIa, and also additional internal changes (outside-in signalling)[7,8].

The number of inhibitors of platelet function is enormous and constantly increasing. In 1991, George and Shattil[9] provided a large list of drugs, foods, spices and vitamins that may cause abnormalities of platelet function. The inhibitory effect of most of these substances has been found by in vitro testing, and there are few reports of bleeding problems, except with aspirin. Some of the drugs that are routinely recommended for inhibition of thrombosis in subjects at risk, or to inhibit platelet aggregation after bypass surgery, percutaneous transluminal coronary angioplasty (PTCA), endarterectomy, transplantation, etc. (for example, aspirin, ticlopidine or clopidogrel) are discussed in Part IV. The most recently developed inhibitors of arterial thrombosis are those that inhibit the interaction of fibrinogen with its receptor, GPIIb/IIIa, on activated platelets[10,11] (Chapter 63). However, any of these antithrombotic agents that interfere with platelet aggregation carry with them the risk of bleeding since platelet aggregation is involved in hemostasis as well as in the formation of arterial thrombi.

A number of naturally occurring, but rare, abnormalities of platelet aggregation have contributed significantly to our understanding of the processes by which platelets adhere to each other. Some of these are considered in Chapters 42–44 and in the chapters concerning platelet receptors (Chapters 8–13). They include Glanzmann's thrombasthenia in which abnormalities of GPIIb/IIIa have been identified[12,13]; von Willebrand disease[14]; the Bernard–Soulier syndrome in which glycoprotein Ib/IX/V is defective[15]; platelet storage pool defects[16,17]; abnormalities of the thromboxane A_2 receptor[18]; disorders of signal transduction and secretion[19]; and defects of the ADP receptor[20–22].

Platelet aggregation in vitro

A number of devices have been used to measure platelet aggregation in vitro (Chapters 30 and 31). Initially, aggregation was observed visually in anticoagulated platelet-rich plasma, or microscopically. The development of aggregometers in which changes in light transmission could be measured at 37 °C in rapidly stirred platelet-rich plasma or in suspensions of washed platelets provided a far greater opportunity for measuring platelet responsiveness to a variety of stimuli and the effect of inhibitors of platelet aggregation[23,24]. More recently, platelet aggregation in diluted whole blood has been studied by impedance aggregometry[25,26]. Other techniques involving quenched-flow single-particle counting devices[27]; platelet function analysers (PFA-100, Dade)[28,29]; and RPFA (rapid platelet function assay, Accumetrics)[30], cone-and-plate devices[31,32], and flow cytometry[33] have been used to assess the extent of platelet aggregation. Some of these methods have been compared by Bennett[34] and by Nicholson and colleagues[35]. Currently, there is intense interest in bedside devices such as the RPFA to monitor platelet reactivity during the administration of inhibitors of GPIIb/IIIa[36,37].

Turbidometric platelet aggregation

After interaction with an aggregating agent, platelets rapidly change shape from discs to spiny spheres with pseudopodia (see Chapter 22). In an aggregometer, this change results in a decrease in light transmission, and a decrease in the amplitude of the oscillations of light transmission that are characteristic of disc-shaped platelets. Shape change can occur without platelet aggregation, for example, if platelets are exposed to an aggregating agent without stirring, or if no fibrinogen or von Willebrand factor is available for binding. In a stirred system, there usually appears to be a lag phase before an increase in light transmission occurs, but small aggregates (2–6 platelets) have been found to be present during this time[38–41]. The formation of larger aggregates is indicated by an increase in light transmission and eventually by large oscillations as the aggregates interrupt the light beam (Fig. 23.1). If the platelets deaggregate, light transmission decreases. Upon stimulation with most aggregating agents (ADP is a major exception, see below), platelets form thromboxane A_2 and release the contents of their storage granules, P-selectin (CD62P, an alpha granule membrane protein) appears on the platelet surface, and the surface develops procoagulant activity (see Chapters 24–26). Strong stimulation, for example with a combination of collagen and thrombin, results in the formation of microparticles[42]. The presence of circulating, procoagulant microparticles has been demonstrated in several clinical conditions[43]. These events are the result of the intracellular signalling reactions that are set in motion when an agonist interacts with its receptor(s) on the platelet surface. Among the internal changes that occur are an increase in cytosolic calcium, activation of phospholipase A_2 leading to mobilization of arachidonic acid which is converted to thromboxane A_2, and activation of phospholipase C, causing breakdown of inositol phospholipids to form 1,2-diacyl glycerol (DAG) and 1,4,5-inositol trisphosphate (IP_3). DAG activates protein kinase C and IP_3 causes mobilization of calcium from internal stores. More detailed descriptions of internal signalling and cytoskeletal changes are given in other chapters in this section.

The extent to which platelets aggregate in vitro is influ-

Cambridge Books Online © Cambridge University Press, 2009

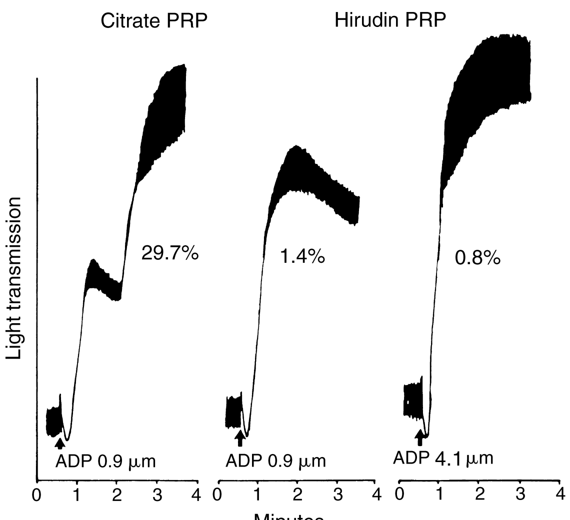


Fig. 23.1. Effect of the anticoagulant on aggregation and release of ^{14}C-serotonin from prelabelled platelets stimulated with ADP. Platelets in citrated plasma (low calcium concentration) undergo two phases of aggregation and release ^{14}C-serotonin from their amine storage granules in response to 0.9 μM ADP (left tracing). Platelets in plasma from blood anticoagulated with hirudin undergo only a single phase of aggregation and do not release amine storage granule contents (centre tracing), even when the concentration of ADP is higher (4.1 μM) (right tracing). The percentages of ^{14}C-serotonin released in three minutes after the addition of ADP are shown beside the aggregation tracings. Reprinted with permission[44].

enced by the conditions under which the blood is collected. Care should be taken to avoid stressing the donor who should not have had antiplatelet drugs during the preceding week; the time between venipuncture and testing should be monitored since the responsiveness of platelets may increase as the time after venipuncture lengthens if CO_2 is lost and the pH rises; hemolysis must be avoided because red blood cells contain ADP; siliconized or plastic tubes should be used to prevent platelets sticking to the sides of the containers; storage temperature, plasma pH, platelet count, length of stir bar and speed of stirring should be standardized. These requirements have been discussed in detail elsewhere[44–47]. When aggregation is to be tested in suspensions of isolated platelets in artificial media, a physiological concentration of ionized calcium (1–2 mM), a source of metabolic energy such as glucose, a protective protein such as albumin, and apyrase to degrade any ADP that is lost from the platelets should all be present to retain platelet sensitivity to low concentrations of weak agonists. Added fibrinogen is also necessary in studies of aggregation by ADP or other weak agonists that do not cause the release of appreciable amounts of the contents of the platelet storage granules.

Impedance platelet aggregometry

In this method, anticoagulated whole blood is diluted 1:1 with saline and warmed to 37 °C in a cuvette in which a two-electrode assembly is inserted[34]. While the sample is stirred, an aggregating agent is added and the increase in impedance (measured in ohms) is recorded as platelets aggregate and adhere to the electrodes. The recording is similar to that resulting from turbidometric aggregation in platelet-rich plasma, except that the lag phase is prolonged to 10 to 30 s and shape change and second phase aggregation are not evident[26]. Inhibitors of platelet aggregation appear to be less effective in this whole blood impedance method[26] and it is affected by the hematocrit[48,49] and the platelet count[50]. The advantages over turbidometric aggregometry are that less blood is required, the preparation of platelet-rich plasma is avoided, and aggregation of lipemic blood samples can be measured[49]. Impedance aggregometry, like turbidometric aggregometry, is insensitive to the formation of small platelet aggregates[34].

Effects of the anticoagulant

The choice of anticoagulant affects the aggregation response. When EDTA is used, the concentration of ionized calcium is too low to support platelet aggregation, although shape change occurs. However, in some individuals, platelets are agglutinated in EDTA-anticoagulated blood by autoantibodies, leading to falsely low platelet counts; this phenomenon was at one time described as spontaneous aggregation, but it is now referred to as pseudothrombocytopenia[51–53]. In contrast, normal platelets that are stored at 37 °C for 5–7 minutes or longer in citrated plasma to which EDTA has been added, lose their ability to aggregate in response to ADP and other weak agonists when adequate concentrations of calcium are restored[54]. This loss of aggregability caused by strong calcium chelation is attributed to an irreversible change in the fibrinogen receptor, GPIIb/IIIa.

Anticoagulants that preserve the physiological concentration of ionized calcium are hirudin (now available as recombinant hirudin) and PPACK (D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone), both of which act by inhibiting the thrombin that forms after blood collection. However, although PPACK is a satisfactory anticoagulant for human blood, it agglutinates rabbit platelets[55]. Heparin has had a long history of use as an anticoagulant,

Cambridge Books Online © Cambridge University Press, 2009

but as Zucker[56] pointed out in 1975 'platelet aggregates are commonly found in blood collected directly into heparin. Their presence can be inferred from the low platelet count that may be noted in platelet-rich plasma'. The platelet aggregates sediment with the red blood cells or stick to the walls of the tube[46].

By far the most commonly used anticoagulant is sodium citrate, which acts by lowering the concentration of ionized calcium into the micromolar range (40 μM when 10.9 mM citrate is used[57] and <5 μM when 12.9 mM citrate is used[58]), thus inhibiting thrombin formation so that aggregation can be tested in platelet-rich plasma. However, this low concentration of ionized calcium has two effects that are seldom understood. The first effect is that, in this low calcium medium, platelets brought into close contact with each other by rapid stirring are induced to form thromboxane A_2, release their granule contents, including ADP from the amine storage (dense) granules, and undergo a second phase of aggregation that is not readily reversible (Fig. 23.1)[59]. This phenomenon occurs not only in citrated platelet-rich plasma, but in suspensions of washed platelets in media to which no calcium salt has been added. In such a medium the concentration of ionized calcium is ≈20 μM and ADP will induce a primary phase of aggregation, followed by the secondary phase (Fig. 23.2), as it does in citrated platelet-rich plasma. However, if calcium (0.5 mM or higher) is added to the medium, only the primary phase of ADP-induced aggregation occurs, thromboxane A_2 does not form and little release of granule contents occurs (Fig. 23.2). Other agents that bring platelets into close contact with each other in a low calcium medium can be shown to have the same effect, such as polylysine[60,61] or the agglutinating agent ristocetin[62]. The secondary phase is abolished by aspirin or other non-steroidal anti-inflammatory drugs which prevent the formation of thromboxane A_2. Because of this inhibition by aspirin, some investigators have chosen to study aggregation in the presence of aspirin to prevent the secondary aggregation effect. However, the primary response of platelets to aggregating agents is diminished in a low calcium medium (compare the extent of primary aggregation in citrated platelet-rich plasma with that in hirudin platelet-rich plasma in Fig. 23.1)[59]. This weaker response is the second, largely unrecognized effect of testing platelet aggregation in a low calcium medium. An important consequence of this effect is the finding that citrate anticoagulation 'enhances the apparent inhibitory activity' of inhibitors that interfere with the interaction of fibrinogen with GPIIb/IIIa, as shown by Phillips and colleagues[63] with Integrilin (eptifibatide). These investigators reported that lower concentrations of Integrilin inhibited platelet aggregation in plasma anticoagulated with citrate

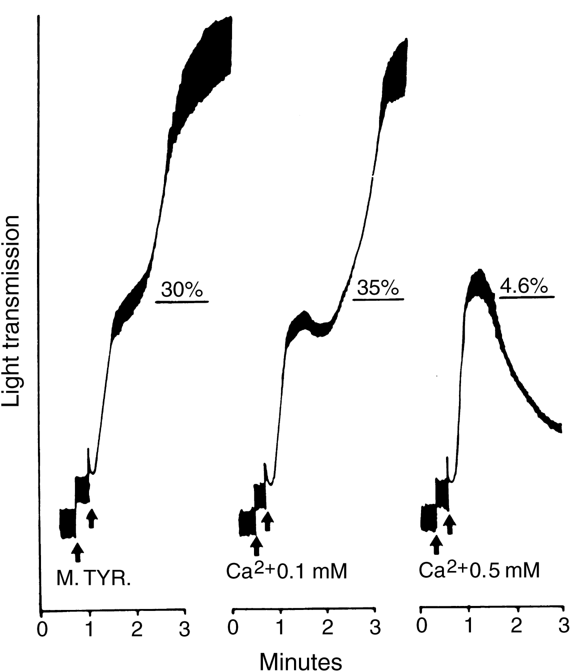


Fig. 23.2. Effect of calcium concentration in the suspending medium of washed human platelets prelabelled with ^{14}C-serotonin and aggregated by ADP (0.8 μM). Platelets were suspended in a modified Tyrode-albumin solution (from which calcium was omitted) containing apyrase and fibrinogen. Without added calcium or with 0.1 mM calcium (left tracings), the platelets undergo two phases of aggregation and release ^{14}C-serotonin (percentage released in two minutes is indicated beside the aggregation curves). In the presence of 0.5 mM calcium, only the primary phase of aggregation occurs and release of ^{14}C-serotonin is greatly inhibited (right tracing).
Reprinted with permission[44].

than with PPACK, and suggested that since the inhibitory activity was overestimated in blood samples collected with citrate, 'it may be possible to achieve greater antithrombotic efficacy beyond that observed in clinical trials to date with Integrilin'. Similar observations have been reported by other investigators[64,65].

Clinically, routine tests of platelet responsiveness to aggregating agents usually involve the use of ADP, collagen, epinephrine, arachidonic acid and ristocetin with platelets in citrated platelet-rich plasma. With platelets from individuals with Glanzmann's thrombasthenia, none of these aggregating agents induces aggregation, but the agglutinating agent ristocetin causes platelets to adhere to each other. Lack of aggregation in response to arachidonic acid and epinephrine, abnormally low responsiveness to collagen, and only a primary phase of aggregation in response to ADP, usually indicates that the subject has taken aspirin

Cambridge Books Online © Cambridge University Press, 2009

or one of the large number of other substances that inhibit thromboxane A_2 formation. (In 1974, Leist and Banwell[66] provided a list of about 250 drugs that contain aspirin.) Rarely would an individual with a defect in the formation of thromboxane A_2[67] or of the thromboxane A_2 receptor(s)[18] be encountered. If ristocetin-induced agglutination is defective, either von Willebrand disease or Bernard–Soulier syndrome is indicated. A more definitive test for von Willebrand disease can be done with fixed or lyophilized platelets[68].

ADP-induced aggregation

In the early days (1964), ADP was administered intravenously to humans[69], but other experiments of this type have been done only with experimental animals[70,71]. ADP injected into the blood stream causes a rapid fall in the platelet count and many platelet aggregates are observable in arterioles, venules and capillaries. After a short time, however, the platelet count returns to the pre-infusion level and few platelet aggregates are visible upon histological examination. Thus, in vivo, ADP-induced aggregation is readily reversible, undoubtedly because only the primary phase of aggregation occurs, and because the ADP phosphohydrolase in plasma[72] and the ecto-ADPase on other blood cells and on the endothelium[73,74] rapidly dephosphorylate the ADP.

The role of ADP in platelet aggregation was first recognized by Gaarder and her colleagues[75] in 1961 when they showed that ADP from red blood cells could cause aggregation. 'Spontaneous' aggregation occurs under conditions of high shear and is much greater in whole blood than in platelet-rich plasma; ADP from red blood cells is at least partly responsible since enzymatic removal of ADP inhibits this effect[76–78]. ADP from red blood cells may be important in the formation of the platelet aggregates in arterial thrombi, particularly at sites of high shear and in conditions in which hemolysis occurs[73].

ADP is also released from stimulated platelets themselves and plays an important synergistic role in aggregation by all agonists that induce the release of platelet granule contents. It also has been shown to take part in stabilizing aggregates induced by other agonists such as thrombin[79,80].

There appear to be three ADP receptors on platelets, namely $P2X_1$, an ion channel linked to Ca^{2+} influx, the purinergic $P2Y_1$ receptor that is responsible for shape change through intracellular calcium mobilization, and a $P2_{cyc}$ ($P2T_{AC}$) receptor that causes adenylyl cyclase inhibition and is involved in enhancing aggregation[12,81]. (See Chapter 9.)

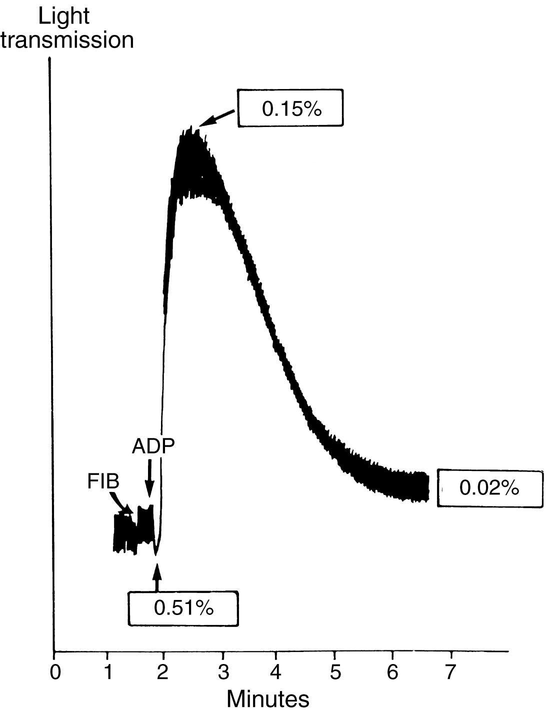


Fig. 23.3. Association of ^{125}I-fibrinogen with washed human platelets during shape change, aggregation and deaggregation induced by 10 μM ADP. Percentages of radioactive fibrinogen associated with the platelets are shown in the boxes beside the aggregation curve.

In vitro, in a medium with a physiological concentration of ionized calcium, ADP causes only a primary phase of platelet aggregation that is readily reversible since it is not accompanied by thromboxane A_2 formation or significant release of platelet granule contents. Little P-selectin appears on the platelet surface and procoagulant activity does not develop. The fibrinogen that binds to the platelets during aggregation dissociates during deaggregation (Fig. 23.3)[82]. Primary aggregation of this type is readily demonstrable in platelet-rich plasma from human blood anticoagulated with hirudin or PPACK, and in suspensions of washed platelets in media containing a physiological concentration of ionized calcium (Figs. 23.1, 23.2). Under these conditions, although high concentrations of ADP cause extensive aggregation, the changes associated with secondary aggregation do not occur (Fig. 23.1). However, as discussed above, when human platelets in a low calcium medium such as citrated platelet-rich plasma are aggregated by ADP at concentrations above 1–2 μM, primary aggregation does not reverse, but is followed by secondary aggregation. At intermediate concentrations of ADP, this secondary response is evident as a second wave of the

Cambridge Books Online © Cambridge University Press, 2009

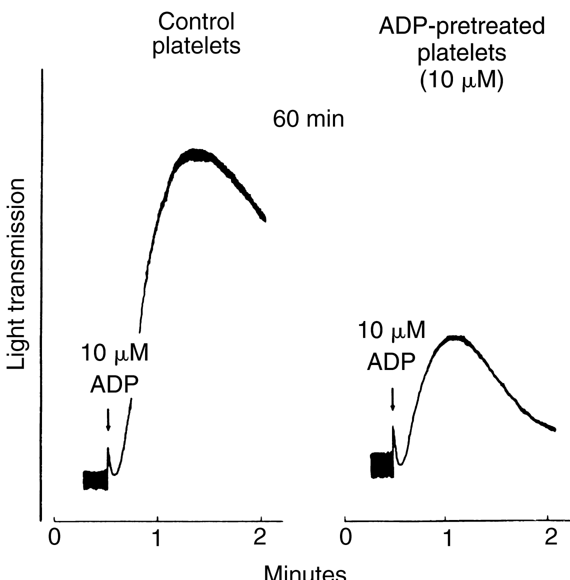


Fig. 23.4. Effect of pretreating platelets with 10 μM ADP on their subsequent response to a second stimulation with ADP. Washed platelets in Tyrode-albumin solution were exposed for 60 minutes to the diluent solution (control, left tracing) or ADP (right tracing) before the addition of fibrinogen and 10 μM ADP.

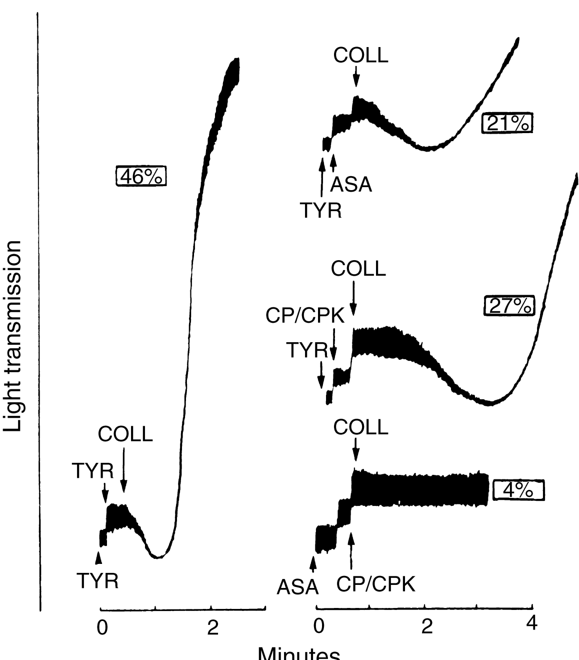


Fig. 23.5. Effect of creatine phosphate/creatine phosphokinase (CP/CPK, to convert released ADP to ATP) and aspirin (ASA, to prevent formation of thromboxane A_2) on platelet aggregation and release of ^{14}C-serotonin from prelabelled platelets. In combination, these inhibitors block the responses of platelets to collagen (COLL). Tyrode solution (TYR) was added to maintain constant volume. The percentages of released ^{14}C-serotonin 3 minutes after the addition of collagen are shown in the boxes beside the aggregation tracings.

increase in light transmission in an aggregometer (Fig. 23.1), but with high ADP concentrations (10 μM or higher) the first and second waves fuse. There is a species difference in the effect of a low concentration of ionized calcium on ADP-induced aggregation. Platelets from humans and most other primates, cats, piebald and rosette guinea pigs (but not white guinea pigs), and about one third of mongrel dogs undergo secondary ADP-induced aggregation, but platelets from rabbits, rats, mice, pigs, mink, cows, goat, sheep, horses and most dogs do not[83–85].

In vitro, platelets that have undergone the primary phase of ADP-induced aggregation and have deaggregated are temporarily refractory to subsequent additions of ADP (Fig. 23.4)[23,86–89]. Platelets that have been isolated and resuspended in an artificial medium also become refractory to ADP. Because the development of this refractoriness can be prevented by the inclusion of apyrase (ATPDase EC 3.6.1.5) in the suspending medium, it is evident that ADP lost from the platelets themselves is responsible for this diminished responsiveness[90,91]. Recently, the refractory state has been attributed to desensitization of the $P2Y_1$ receptor caused by its internalization[89].

One to two days following their oral or intravenous administration, the thienopyridines ticlopidine and clopidogrel (actually their metabolites) specifically inhibit ADP-induced aggregation[92,93]. This inhibition has been attributed to their blocking the inhibitory effect of ADP on adenylyl cyclase[94,95]. (See Chapter 62.)

Aggregation by collagen

Several components of the platelet surface have been implicated as receptors for collagen[96,97]. (See Part 1, Chapter 11.) Although at least 19 types of collagen have been characterized, types I and III are most effective as platelet aggregating agents. The native triple–helical structure of collagen is required[98] and usually fibrils from equine tendon are used. GPIa/IIa (integrin $\alpha2\beta1$) and GPVI are the receptors on platelets through which they adhere to collagen and are activated[12].

When collagen is tested as a platelet aggregating agent in vitro, there may be a lag phase of up to a minute before there is an appreciable increase in light transmission in an aggregometer (Fig. 23.5). During this time some of the platelets adhere to the collagen fibrils and are induced to

Cambridge Books Online © Cambridge University Press, 2009

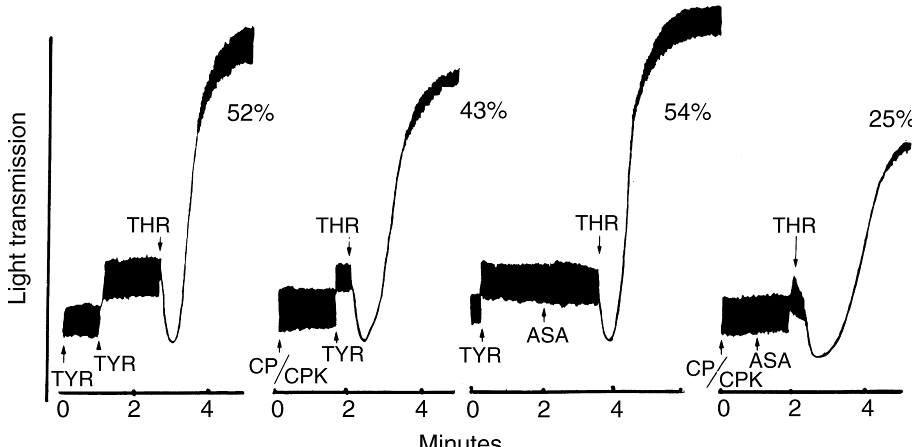


Fig. 23.6. Effect of creatine phosphate/creatine phosphokinase (CP/CPK, to convert released ADP to ATP) and aspirin (ASA, to prevent formation of thromboxane A_2) on platelet aggregation and release of ^{14}C-serotonin from prelabelled platelets. This combination of inhibitors reduces, but does not abolish, platelet responses to 0.05 U/ml thrombin (THR). Tyrode solution (TYR) was added to maintain constant volume. The percentages of released ^{14}C-serotonin three minutes after the addition of thrombin are shown in the boxes beside the aggregation tracings.

form thromboxane A_2 and to release ADP. These two aggregating agents act synergistically on the non-adherent platelets, causing aggregation. Aggregation can be lessened by inhibiting either agonist, for example, by the use of aspirin to prevent formation of thromboxane A_2, or by rapid removal of ADP with apyrase or creatine phosphate/creatine phosphokinase (CP/CPK); the combination of these two types of inhibitors may be completely inhibitory (Fig. 23.5)[99,100].

Aggregation induced by arachidonic acid and thromboxane A2

When platelets interact with most aggregating agents, phospholipase A_2 is activated and liberates arachidonic acid from membrane phospholipids. The cyclo-oxygenase and thromboxane synthase enzymes in the platelets convert arachidonic acid to thromboxane A_2[101] which causes aggregation and the release of platelet granule contents (Chapter 10). Non-steroidal anti-inflammatory drugs inhibit cyclo-oxygenase, but aspirin is unique in that it irreversibly acetylates the enzyme so that it is inhibited for the remainder of the life of the platelet. Thus the effect of the ingestion of aspirin lasts for several days and is indicated by absent or diminished responsiveness of the platelets to arachidonic acid. Since thromboxane A_2 is very short-lived, tests of it as an aggregating agent are generally done with stable mimetics, such as U46619 (9,11 dideoxy-11α-epoxymethano-prostaglandin $F_{2\alpha}$)[102].

Some drugs with both thromboxane synthase inhibitory activity and thromboxane receptor-blocking effects have been developed to prevent the activation of platelets by thromboxane A_2[103].

Thrombin-induced aggregation

Thrombin is the strongest aggregating agent that platelets encounter in vivo. As described in Chapter 8, the protease-activated receptors, PAR1 and PAR4, are responsible for the induction of aggregation by thrombin[104]. Although thrombin causes the formation of thromboxane A_2 and releases ADP from the platelet dense granules, and these aggregating agents increase the extent of thrombin-induced aggregation, it is not prevented when thromboxane A_2 formation is blocked and ADP is rapidly removed (Fig. 23.6)[99,100]. However, ADP has been shown to stabilize thrombin-induced aggregates[79,80].

Although thrombin-induced aggregation cannot be studied satisfactorily in platelet-rich plasma because of the formation of fibrin, the effects of thrombin are readily studied in suspensions of washed platelets in artificial media. After the thrombin receptor, PAR1, and its mechanism of action were discovered by Coughlin's group[105], peptides of the tethered ligand such as SFLLRN (also called TRAP, i.e. thrombin receptor-activating peptide) have been used to study aggregation in platelet-rich plasma as well as in suspensions of washed platelets. In using such peptides as surrogates for thrombin, one must be aware that they do

Cambridge Books Online © Cambridge University Press, 2009

not interact with other thrombin receptors such as PAR4 or the high affinity thrombin receptor on glycoprotein Ib. In addition, SFLLRN does not aggregate platelets from rabbits, rats, dogs, pigs and hamsters[106,107].

Unexpectedly, Furman and colleagues[108] have shown an aggregating effect on human platelets of the peptide that is cleaved from the thrombin receptor when the tethered ligand is exposed.

When platelets have been aggregated by thrombin, they are not readily deaggregated, because fibrin formed from fibrinogen released from the alpha-granules binds them together; it is necessary to use a proteolytic enzyme such as chymotrypsin or plasmin to achieve deaggregation[109,110]. (In vivo, fibrinolytic agents are effective in disrupting arterial thrombi.) It is, however, possible to activate platelets with thrombin to induce them to release their granule contents without aggregation and recover them as discrete, degranulated platelets[111]. Degranulation with SFLLRN has been developed as an even gentler method[112]. Degranulated platelets have P-selectin and procoagulant phospholipid on their surface. Despite the reactions that they have undergone, in experiments with rabbits and baboons, degranulated platelets have been shown to survive normally upon reinjection[109,113]. P-selectin is lost from the surface of thrombin-activated baboon platelets in the circulation[113], but the length of time that they retain their procoagulant activity in vivo is not established (in vitro it is present for up to 4 hours[114]). If the procoagulant surface persists for an appreciable time in vivo, platelets that recirculate following disruption of a thrombus by fibrinolysis and the forces of flowing blood may contribute to thrombin generation and further aggregation at other sites in the circulation.

Epinephrine

Although epinephrine is not a true aggregating agent[3–5], it does aggregate platelets in citrated platelet-rich plasma[115]. However, an initial change in platelet shape does not occur[3,47,86,116]. Epinephrine rarely aggregates platelets in plasma from blood anticoagulated with hirudin or PPACK (Fig. 23.7)[3,57]. Epinephrine-induced aggregation in citrated platelet-rich plasma is invariably inhibited if subjects have recently ingested aspirin or other drugs that prevent thromboxane A_2 formation, providing an excellent test for the presence of such inhibitors.

Epinephrine interacts with α_2-adrenergic receptors on human platelets, synergistically and strongly enhancing the effects of other aggregating agents, even when they are tested at concentrations too low to have measurable aggregating effects by themselves[1,3]. The epinephrine-induced aggregation in citrated platelet-rich plasma is probably the result of traces of ADP and/or thrombin[4,117]. It seems likely that the wide variations in the responsiveness of human platelets to epinephrine is related to the concentration of other agonists in the plasma, and/or the presence of inhibitors from ingested substances.

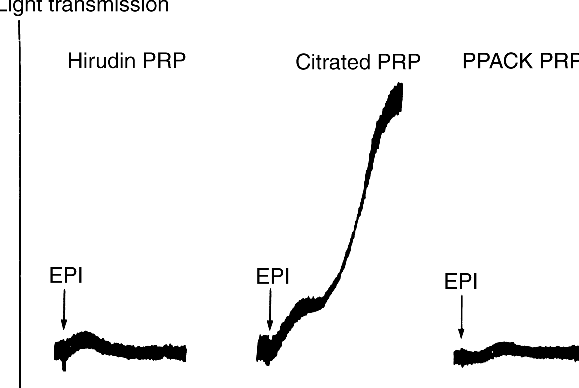


Fig. 23.7. Effect of 10 μM epinephrine (EPI) in platelet-rich plasma (PRP) from blood anticoagulated with hirudin (50 U/ml), sodium citrate (1 part 3.8% to 9 parts of blood), or PPACK ($FPRCH_2Cl$, 40 μM). Platelets maintain their disc shape, indicated by the oscillations of light transmission, and extensive aggregation occurs only in citrated PRP. (Reprinted with permission[3].)

It has been suggested that stress, leading to elevated levels of circulating epinephrine, may be linked to coronary heart disease[118,119].

Aggregation induced by platelet activating factor (PAF)

The wave of interest in PAF (1–O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) as a platelet aggregating agent[120] has passed and its other functions have been recognized as of more importance[121]. PAF originates from IgE-sensitized basophils, stimulated macrophages and neutrophils, and platelets themselves[122], although human platelets, in contrast to rabbit platelets, form very little upon stimulation with thrombin[123]. Human platelets are much less sensitive to PAF than are guinea pig or rabbit platelets with which much of the early work was done; platelets from rats, mice and most primates are insensitive to PAF[120,124]. At one time PAF was considered as possibly responsible for a postulated third pathway of thrombin-induced aggregation, but with

Cambridge Books Online © Cambridge University Press, 2009

the identification and elucidation of the action of the thrombin receptor, PAR1, this suggestion regarding the role of PAF is no longer considered.

Nevertheless, PAF is a weak aggregating agent, similar to ADP in that in a medium with a physiological concentration of calcium it does not cause thromboxane A_2 formation or extensive release of granule contents. However, in citrated platelet-rich plasma or an artificial medium with no added calcium, PAF induces human platelets to form thromboxane A_2 and release ADP[120]. There may be circumstances in vivo in which PAF acts synergistically with other agonists to enhance aggregation.

Serotonin-induced aggregation

Serotonin (5-hydroxytryptamine, 5-HT) is a weak agonist for human platelets and the slight aggregation response is followed by deaggregation[115,125]. In contrast, cat platelets in citrated platelet-rich plasma undergo second phase aggregation in response to serotonin at concentrations greater than 3 μM[126]. Serotonin exerts mild synergism with other agonists[1,127]. Platelet activation by serotonin involves 5-HT_2 receptor binding sites which differ from the sites responsible for serotonin uptake[128] as shown by experiments with ketanserin, a selective 5-HT_2 receptor antagonist.[127,129]. Platelets carry all the serotonin in the blood in their amine storage granules and when it is released upon stimulation with agonists such as thrombin or collagen, it enhances their effects.

Aggregation induced by arginine vasopressin (AVP)

Arginine vasopressin is a nine amino acid peptide hormone with effects on many systems in addition to its effect on platelets. Wun[130] has reviewed the evidence that the receptor on human platelets is of the V_{1a} type.

In 1972, Haslam and Rosson[131] discovered that platelets are aggregated by vasopressin, although it is classified as a 'weak' agonist. A concentration of at least 2.5 nM is required for minimal aggregation, with a maximum response at 100 nM, and this concentration is several orders of magnitude greater than physiological levels. However, in heparinized platelet-rich plasma, 1 pM has been shown to cause the appearance of P-selectin on the platelet surface, indicating activation of the platelets[132]. In addition, vasopressin can act in synergy with other aggregating agents[118,133]. The aggregation response of human platelets to vasopressin has been shown to be heterogeneous[134]. Platelets from some normal individuals display only primary aggregation, followed by deaggregation, induced by concentrations of vasopressin as high as 100 nM, whereas platelets from other subjects undergo extensive secondary aggregation in response to as little as 10 nM[134].

The beneficial effect of vasopressin infusions in gastrointestinal bleeding may be related to a combination of vasoconstriction with the release of von Willebrand factor, and platelet activation[130].

The synthetic analogue of vasopressin, Desmopressin (1-desamino-8-D-arginine vasopressin, DDAVP) has not been shown to aggregate platelets, but it does increase platelet aggregation induced by collagen or ADP[135]. DDAVP primarily acts on V_2 receptors and has only minor V_1 agonist activity[130]. However, in concentrations reached after the usual therapeutic dose, DDAVP does induce the expression of P-selectin on the platelet surface[136].

Thrombospondin-induced aggregation

The interactions of thrombospondin with platelets have been reviewed by Mosher[137] and by Legrand and coworkers[138]. It is released from the alpha granules of stimulated platelets and binds to the platelet surface; it is also present in the subendothelial matrix. Thrombospondin is responsible for the endogenous lectin activity of stimulated platelets[139]. It has been suggested that thrombospondin binds to fibrinogen and to the platelet surface and thus strengthens fibrinogen binding[140]. In vitro, thrombospondin has been reported to slowly aggregate platelets in a reaction involving GPIV (CD36), but not GPIIb/IIIa[141]. It also promotes aggregation induced by other agonists. Mosher[137] has pointed out that aggregated thrombospondin could agglutinate platelets, and that there are many possible interactions of thrombospondin with molecules at the platelet surface. Recently, thrombospondin has been shown to activate platelets via integrin-associated protein (IAP/CD47), a five membrane-spanning receptor[142]. Chung and colleagues[143] have raised the possibility of a role for IAP and thrombospondin in the early activation of platelets upon adhesion to collagen.

Aggregation induced by cathepsin G

The proteolytic enzyme cathepsin G from activated leukocytes can induce platelet aggregation, release of granule contents, thromboxane A_2 formation, increase in cytosolic calcium and activation of protein kinase C[144–146]. In 1994, Selak[147] obtained evidence that the receptor for cathepsin

Cambridge Books Online © Cambridge University Press, 2009

G differed from the receptor for thrombin, and it has now been shown that the protease-activated receptor, PAR4, mediates the responses of platelets to cathepsin G[148]. Cathepsin G also has synergistic effects with other aggregating agents and these effects are partly due to released ADP[149]. Pretreatment of platelets with this enzyme cleaves the thrombin receptor PAR1 so that the tethered ligand is lost and the platelet responses to thrombin are greatly reduced. Their responsiveness to some other aggregating agents (collagen, SFLLRN, U46619) is diminished, but they remain normally responsive to ADP in the presence of fibrinogen[150]. Thus, although cathepsin G activates platelets, if they recirculate after interaction with it, their subsequent responses to aggregating agents will be lessened.

Effects of plasmin

The proteolytic enzyme plasmin cleaves GPIb and GPIIb/IIIa on the platelet surface, in addition to its fibrinolytic action[151–153]. In vivo, platelets are exposed to endogenous plasmin, but they are also exposed to high concentrations when plasminogen activators are infused to disrupt thrombi in arteries[154]. When platelets that have been exposed to plasmin during fibrinolysis return to the circulation, their subsequent responses to aggregating agents are changed. Coller[154] has reviewed the platelet-activating and platelet-inhibiting effects of fibrinolytic agents in vivo.

In vitro, plasmin at concentrations ≥1 caseinolytic unit causes platelet aggregation, release of granule contents, an increase in cytosolic calcium, inositol phospholipid breakdown and activation of protein kinase C[155,156]. Lower concentrations of plasmin have been reported to inhibit platelet responses to thrombin and other agonists[157–159]. Experimentally, the effects of plasmin on platelets are affected by its concentration and the time and temperature of incubation of the platelets with plasmin[160]. Plasmin has been reported to activate platelets in the same way as thrombin, cleaving PAR1 at Arg 41 and thus revealing the tethered ligand, SFLLRN[161]. However, pretreatment of platelets with plasmin reduces their responsiveness to thrombin[157,158,162]. It was suggested and later shown[163] that plasmin cleaves PAR1 at a point distal to Arg 41 so that SFLLRN is lost (PAR4 could also be a target for plasmin on human platelets.)

Plasmin causes limited cleavage of GPIIb/IIIa, leading to a conformational change that converts it to the receptor for fibrinogen and von Willebrand factor[153,164]. Consequently, when plasmin-pretreated platelets are stirred in the presence of fibrinogen, they are aggregated/agglutinated. Rabhi-Sabile and colleagues[164] have implicated von Willebrand factor rather than fibrinogen in this process. The observed potentiation of aggregation of plasmin-pretreated platelets by agonists other than thrombin[162] is probably due to increased binding of ligands that interact with partially cleaved GPIIb/IIIa.

Other in vivo aggregating agents

Although high concentrations (>1 μM) of PGE_2 inhibit human platelet aggregation, lower concentrations in the range produced by activated platelets potentiate aggregation by most agonists[165]. There is evidence that this potentiation involves the 'priming of protein kinase C to activation by other agonists'[166]. Interestingly, in heparinized platelet-rich plasma, PGE_2 directly aggregates pig platelets[165].

Beta-amyloid peptides aggregate platelets[167], and augment ADP-induced aggregation[168]. Stromal cell-derived factor 1 and macrophage-derived chemokine are weak agonists, but enhance platelet responses to other aggregating agents[169]. Thrombopoietin potentiates aggregation induced by other agonists[170,171].

It has been suggested that zinc ions may be a requirement for ADP-induced aggregation by activating protein kinase C and enhancing fibrinogen receptor exposure[172,173]. Short-term zinc deficiency impairs platelet aggregation induced by ADP or arachidonate[174].

Many bacteria induce platelet aggregation and the release of granule contents in vitro and in vivo, and the endotoxin formed by gram negative bacteria can cause disseminated intravascular coagulation (DIC) (see Chapter 51). The very weak effects of endotoxin on human platelets are in contrast to the strong effects of endotoxin on platelets from other species, particularly rabbits.

Several viruses have been shown to cause platelet aggregation and the release of granule contents (see Chapter 52).

Some parasites and tumour cells have also been found to aggregate platelets (see Chapters 53 and 54).

Immune complexes can cause platelet aggregation[175,176] and some platelet antibodies also have this effect. Heparin-induced thrombocytopenia (HIT) is caused by IgG antibodies that recognize complexes of heparin and platelet factor 4, resulting in platelet aggregation via platelet Fc gamma IIa receptors[177,178]. Procoagulant microparticles from the stimulated platelets generate thrombin in vivo, which may be largely responsible for thrombosis associated with HIT[179] (see Chapter 39).

Cambridge Books Online © Cambridge University Press, 2009

Shear-induced aggregation

Shear-induced platelet aggregation is thought to be responsible for the formation of arterial thrombi at sites where vessel walls have been narrowed by atherosclerotic plaques[180,181]. High wall shear rates also occur during bleeding from severed arterioles[182].

In vitro, platelets aggregate without the addition of an agonist when they are subjected to high levels of fluid shear stress in devices such as a cone-and-plate viscometer[31]. This aggregation response requires the presence of von Willebrand factor multimers, extracellular calcium, ADP released from the platelets, and GPIb and GPIIb/IIIa on the platelet membrane[183]. Shear stress-induced aggregation does not depend on the formation of thromboxane A_2 and thus is not inhibited by aspirin.

Evidence has been obtained that during thrombus formation under high shear stress, von Willebrand factor binds to GPIb and to GPIIb/IIIa and the thrombi are stabilized by fibrinogen binding to GPIIb/IIIa on the activated platelets[184–186]. Shear-induced platelet aggregation is enhanced in platelet-rich plasma from patients with acute myocardial infarction, apparently as a result of an increase in the concentration of von Willebrand factor in the plasma[187,188] (see Part I, Chapter 13).

Spontaneous aggregation and platelet hypersensitivity

In some conditions, spontaneous aggregation occurs upon stirring anticoagulated blood or platelet-rich plasma. Peerschke and Zucker[189] observed spontaneous aggregation upon rewarming and shaking chilled platelets in the presence of fibrinogen. Spontaneous aggregation is a feature of the Montreal Platelet Syndrome[190]. Triplett and colleagues[45] listed a number of conditions in which spontaneous aggregation had been observed, including transient cerebral ischemia, angina pectoris, myocardial infarction, acute peripheral vascular occlusion, and diabetes. Platelet hypersensitivity to aggregating agents has also been reported in these conditions and in hyperbetalipoproteinemia, paroxysmal nocturnal hemoglobinuria, migraine, gout, acute renal failure and hypertension[191,192].

Trip and colleagues[193] have indicated that spontaneous platelet aggregation in vitro is a useful biologic marker for prediction of coronary events and mortality in survivors of myocardial infarction. However, sensitivity of platelets to ADP-induced aggregation does not appear to be predictive of the risk of first episodes of ischemic heart disease[194].

Agglutination induced by ristocetin or botrocetin

The antibiotic ristocetin induces agglutination rather than aggregation, involving von Willebrand factor forming bridges between the platelets. In tests of von Willebrand factor activity, ristocetin is used with paraformaldehyde-fixed platelets[68]. Botrocetin, derived from a snake venom, is used in a similar manner. Unlike aggregation, agglutination does not depend on the metabolic capability of the platelets. When ristocetin is added to unfixed platelets in stirred, citrated platelet-rich plasma, agglutination is followed by a second phase that is aggregation caused by thromboxane A_2 and released ADP.[62].

Bovine or porcine von Willebrand factor causes agglutination of human platelets without the addition of ristocetin[195,196].

Endogenous inhibitors of platelet aggregation

Foremost among inhibitors of aggregation that platelets encounter in the circulation are those that stimulate an increase in the concentration of cyclic AMP in platelets (see Part I, Chapter 20). Adenosine, arising from the dephosphorylation of ATP and ADP, is one of these[197]. With platelets from humans, sheep, dogs and rabbits, adenosine is a potent inhibitor, but it is less effective with platelets from horses, guinea pigs, cats, rats and mice.

Normally, adenosine formed in plasma is rapidly taken up by red blood cells, but this uptake is blocked by the vasodilator dipyridamole, and related pyrimido-pyrimidines. Although these drugs can also raise cyclic AMP levels by inhibition of phosphodiesterase, it is considered that they exert most of their inhibitory effects on platelets by increasing the concentration of adenosine in plasma[198].

Prostacyclin (PGI_2), produced from arachidonic acid by endothelial cells, also raises the concentration of cyclic AMP in platelets and is a potent inhibitor of platelet aggregation[199]. Prostaglandin D_2 is also an inhibitor formed in vivo in small amounts[200].

The vasodilator nitric oxide (NO, originally called endothelium-derived relaxing factor) is a short-lived inhibitor that acts by increasing the concentration of cyclic GMP in platelets[201]. It is formed by the endothelium, platelets and leukocytes[202,203].

ATP and AMP inhibit ADP-induced aggregation by interfering with the binding of ADP to its receptors[204]. ADP is broken down by phosphohydrolases in plasma[72] and by the ecto ADPase on other blood cells and on the endothelium[73,74].

Cambridge Books Online © Cambridge University Press, 2009

Antithrombin (antithrombin III) inhibits the action of thrombin, particularly in the presence of heparin[205]. Heparin cofactor II also inhibits thrombin, and tissue factor pathway inhibitor (TFPI) interferes with the generation of thrombin[206]. The protein C anticoagulant pathway also reduces thrombin formation; its action involves the combination of thrombin with thrombomodulin to activate protein C. Activated protein C forms a complex with plasma protein S that inactivates coagulation factors Va and VIIIa on membrane surfaces[207].

Non-physiological aggregating agents used only in vitro

A large number of reagents that aggregate platelets have been used in vitro in studies of the mechanisms involved in aggregation[204,208], but a comprehensive list of these is beyond the scope of this review. Only a few of the most commonly used agonists will be mentioned here.

The calcium ionophores A23187 and ionomycin induce shape change, aggregation, and the release of granule contents[209]. They have been used extensively to investigate the effects of raising the concentration of calcium in platelets. A23187 is a strong stimulus for microparticle formation[42].

The tumour-promoting agent, phorbol myristate acetate, is an aggregating agent[210] that exerts its effect by activating protein kinase C[211]. Diacylglycerols have a similar effect[212].

Sodium fluoride and fluoroaluminate (AlF_4) have been used to explore G-protein mediated platelet responses[213–215].

Since thromboxane A_2 is a very short-lived compound, several more stable mimetics, such as U46619 are used to stimulate the thromboxane A_2 receptor[102].

Thrombin receptor activating peptides (TRAP), such as SFLLRN or the 14 amino acid peptide that contains it, stimulate the thrombin receptor, PAR1, but do not bind to the other thrombin receptors on platelets[104].

Abnormalities of platelet aggregation

A number of reviews are available that describe hereditary and acquired conditions associated with decreased platelet aggregation and those associated with increased platelet aggregation[12,19,45,216]. Acquired abnormalities of platelet aggregation can be subdivided into those acquired because of disease, and those acquired because of drugs and other ingested substances[9]. The recent increased use of herbal medicines will undoubtedly add to the list of agents that influence platelet function in vivo.

Currently there is considerable interest in ethanol which is a weak inhibitor of aggregation induced by some agonists[217]. This interest has arisen because of the epidemiological evidence that moderate consumption of ethanol is associated with a reduction in the thromboembolic complications of coronary artery disease[218].

Part III has a number of chapters concerning platelet abnormalities, both inherited and acquired.

Conclusion

Platelet aggregation plays a significant role in hemostasis and thrombosis, particularly arterial thrombosis. A wide variety of agents cause platelet aggregation or contribute to its extent. A very large number of inhibitors have been identified and many new ones have been developed. The complexities of receptors and the signalling pathways in platelets are still unfolding. As a result of increased understanding of the mechanisms involved in aggregation, several therapeutic interventions are in use that have significantly reduced the morbidity and mortality from hemorrhagic and thrombotic disorders.

References

1 Kinlough-Rathbone RL, Mustard JF. Synergism of agonists. In Holmsen H, ed. *Platelet responses and metabolism.* Boca Raton, FL: CRC Press; 1986: 193–207.

2 Ware JA, Smith M, Salzman EW. Synergism of platelet-aggregating agents. Role of elevation of cytoplasmic calcium. *J Clin Invest* 1987; 80: 267–271.

3 Lalau Keraly C, Kinlough-Rathbone RL, Packham MA, Suzuki H, Mustard JF. Conditions affecting the responses of human platelets to epinephrine. *Thromb Haemost* 1988; 60: 209–216.

4 Lanza F, Beretz A, Stierlé A, Hanau D, Kubina M, Cazenave J-P. Epinephrine potentiates human platelet activation but is not an aggregating agent. *Am J Physiol* 1988; 255: H1276–H1288.

5 Steen VM, Holmsen H, Aarbakke G. The platelet-stimulating effect of adrenaline through a_2-adrenergic receptors requires simultaneous activation by a true stimulatory platelet agonist. Evidence that adrenaline *per se* does not induce human platelet activation in vitro. *Thromb Haemost* 1993; 70: 506–513.

6 Du X, Ginsberg MH. Integrin $\alpha_{IIb}\beta_3$ and platelet function. *Thromb Haemost* 1997; 78: 96–100.

7 Frelinger AL III, Lam SC-T, Plow EF, Smith MA, Loftus JC, Ginsberg MH. Occupancy of an adhesive glycoprotein receptor modulates expression of an antigenic site involved in cell adhesion. *J Biol Chem* 1988; 263: 12397–12402.

Cambridge Books Online © Cambridge University Press, 2009

8 Shattil SJ. Signaling through platelet integrin αIIbβ3: inside-out, outside-in, and sideways. *Thromb Haemost* 1999; 82: 318–325.
9 George JN, Shattil SJ. The clinical importance of acquired abnormalities of platelet function. *N Engl J Med* 1991; 324: 27–39.
10 Coller BS. GPIIb/IIIa antagonists: Pathophysiologic and therapeutic insights from studies of c7E3 Fab. *Thromb Haemost* 1997; 78: 730–735.
11 Tscheng JE. Clinical challenges of platelet glycoprotein IIb/IIIa receptor inhibitor therapy. Bleeding, reversal, thrombocytopenia, and retreatment. *Am Heart J* 2000; 139: S38–S45.
12 Nurden AT. Inherited abnormalities of platelets. *Thromb Haemost* 1999; 82: 468–480.
13 French DL, Seligsohn U. Platelet glycoprotein IIb/IIIa receptors and Glanzmann's thrombasthenia. *Arterioscler Thromb Vasc Biol* 2000; 20: 607–610.
14 Sadler JE, Mannucci PM, Berntorp E et al. Impact, diagnosis and treatment of von Willebrand disease. *Thromb Haemost* 2000; 84: 160–174.
15 López JA, Andrews RK, Afshar-Kharghan V, Berndt MC. Bernard–Soulier syndrome. *Blood* 1998; 91: 4397–4418.
16 Nieuwenhuis HK, Akkerman J-WN, Sixma JJ. Patients with a prolonged bleeding time and normal aggregation tests may have storage pool deficiency: studies on 106 patients. *Blood* 1987; 70: 620–623.
17 Weiss HJ. Inherited abnormalities of platelet granules and signal transduction. In Colman RW, Hirsh J, Marder VJ, Salzman EW, eds. *Hemostasis and thrombosis: basic principles and clinical practice,* 3rd edn. Philadelphia: JB Lippincott; 1993: 673–684.
18 Higuchi W, Fuse I, Hattori A, Aizawa Y. Mutations of the platelet thromboxane A_2 (TXA_2) receptor in patients characterized by the absence of TXA_2-induced platelet aggregation despite normal TXA_2 binding activity. *Thromb Haemost* 1999; 82: 1528–1531.
19 Rao AK. Congenital disorders of platelet function: Disorders of signal transduction and secretion. *Am J Med Sci* 1998; 316: 69–76.
20 Cattaneo M, Lecchi A, Randi AM, McGregor JL, Mannucci PM. Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. *Blood* 1992; 80: 2787–2796.
21 Nurden P, Savi P, Heilmann E, Bihour C, Herbert JM, Maffrand JP, Nurden A. An inherited bleeding disorder linked to a defective interaction between ADP and its receptor on platelets. Its influence on glycoprotein IIb–IIIa complex function. *J Clin Invest* 1995; 95: 1612–1622.
22 Cattaneo M, Gachet C. ADP receptors and clinical bleeding disorders. *Arterioscler Thromb Vasc Biol* 1999; 19: 2281–2285.
23 Born GVR, Cross MJ. The aggregation of blood platelets. *J Physiol* 1963; 168: 178–195.
24 O'Brien, JR. Some effects of adrenaline and anti-adrenaline compounds on platelets in vitro and in vivo. *Nature* 1963; 200: 763–764.
25 Cardinal DC, Flower RJ. The electronic aggregometer: a novel device for assessing platelet behavior in blood. *J Pharmacol Methods* 1980; 3: 135–158.
26 Ingerman-Wojenski C, Smith JB, Silver MJ. Evaluation of electrical aggregometry: Comparison with optical aggregometry, secretion of ATP, and accumulation of radiolabeled platelets. *J Lab Clin Med* 1983; 101: 44–52.
27 Gear AR. Rapid reactions of platelets studied by a quenched-flow approach: aggregation kinetics. *J Lab Clin Med* 1982; 100: 866–886.
28 Rand ML, Carcao MD, Blanchette VS. Use of the PFA-100® in the assessment of primary, platelet-related hemostasis in a pediatric setting. *Semin Thromb Haemost* 1998; 24: 523–529.
29 Fressinaud E, Veyradier A, Truchaud F, Martin I, Boyer-Neumann C, Trossaert M, Meyer D. Screening for von Willebrand disease with a new analyzer using high shear stress: a study of 60 cases. *Blood* 1998; 91: 1325–1331.
30 Storey RF, May JA, Wilcox RG, Heptinstall S. A whole blood assay of inhibition of platelet aggregation by glycoprotein IIb/IIIa antagonists: comparison with other aggregation methodologies. *Thromb Haemost* 1999; 82: 1307–1311.
31 Hardwick RA, Hellums JD, Peterson DM, Moake JL, Olson JD. The effect of PGI_2 and theophylline on the response of platelets subjected to shear stress. *Blood* 1981; 58: 678–681.
32 Varon D, Dardik R, Shenkman B et al. A new method for quantitative analysis of whole blood platelet interaction with extracellular matrix under flow conditions. *Thromb Res* 1997; 85: 283–294.
33 Michelson AD, Barnard MR, Krueger LA, Frelinger AL III, Furman MI. Evaluation of platelet function by flow cytometry. *Methods* 2000; 21: 259–270.
34 Bennett JS. Platelet aggregation. In Beutler E, Lichtman MA, Coller BS, Kipps TJ, eds. *Williams hematology,* 5th ed. New York: McGraw-Hill; 1995: L114–L117.
35 Nicholson NS, Panzer-Knodle SG, Haas NF et al. Assessment of platelet function assays. *Am Heart J* 1998; 135: S170–S178.
36 Li CKN, Hoffmann TJ, Hsieh P-Y, Malik S, Watson WC. The Xylum Clot Signature Analyzer®: a dynamic flow system that simulates vascular injury. *Thromb Res* 1998; 92 (Suppl. 2): S67–S77.
37 Coiffic A, Cazes E, Janvier G et al. Inhibition of platelet aggregation by abciximab but not by aspirin can be detected by a new point-of-care test, the Hemostatus. *Thromb Res* 1999; 95: 83–91.
38 Born GVR, Hume M. Effects of the numbers and sizes of platelet aggregates on the optical density of plasma. *Nature* 1967; 215: 1027–1029.
39 Gear ARL. Reduction in single platelets during primary and secondary aggregation. *Thromb Haemost* 1981; 45: 298.
40 Frojmovic M. ADP-induced binding of fibrinogen to activated platelets is rapid and parallels platelet microaggregation. *Platelets* 1997; 8: 189.
41 Nakamura T, Ariyoshi H, Kambayashi J et al. Effect of low con-

Cambridge Books Online © Cambridge University Press, 2009

centrations of epinephrine on human platelet aggregation analyzed by particle counting method and confocal microscopy. *J Lab Clin Med* 1997; 130: 262–270.
42 Pasquet JM, Dachary-Prigent J, Nurden AT. Microvesicle release is associated with extensive protein tyrosine dephosphorylation in platelets stimulated by A23187 or a mixture of thrombin and collagen. *Biochem J* 1998; 333: 591–599.
43 Solum NO. Procoagulant expression in platelets and defects leading to clinical disorders. *Arterioscler Thromb Vasc Biol* 1999; 19: 2841–2846.
44 Kinlough-Rathbone RL, Packham MA, Mustard JF. Platelet aggregation. In Harker LA, Zimmerman TS, eds. *Methods in hematology. measurements of platelet function.* New York: Churchill Livingstone; 1983: 64–91.
45 Triplett DA, Harms CS, Newhouse P, Clark C. In Triplett DA, ed. *Platelet function, laboratory evaluation and clinical application.* Chicago: Educational Products Division, American Society of Clinical Pathologists; 1978.
46 Zucker MB. Platelet aggregation measured by the photometric method. *Methods Enzymol* 1989; 169: 117–133.
47 White MM, Jennings LK. *Platelet protocols. Research and clinical laboratory procedures.* San Diego, CA: Academic Press; 1999.
48 Mackie IJ, Jones R, Machin SJ. Platelet impedance aggregation in whole blood and its inhibition by antiplatelet drugs. *J Clin Pathol* 1984; 37: 874–878.
49 Galvez A, Badimon L, Badimon J-J, Fuster V. Electrical aggregometry in whole blood from human, pig and rabbit. *Thromb Haemost* 1986; 56: 128–132.
50 Sweeney JD, Labuzetta JW, Fitzpatrick JE. The effect of the platelet count on the aggregation response and adenosine triphosphate release in an impedance lumi-aggregometer. *Am J Clin Pathol* 1988; 89: 655–659.
51 Gowland E, Kay HEM, Spillman JC, Williamson JR. Agglutination of platelets by a serum factor in the presence of EDTA. *J Clin Pathol* 1969; 22: 460–464.
52 Schrezenmeier H, Müller H, Gunsilius E, Heimpel H, Seifried E. Anticoagulant-induced pseudothrombocytopenia and pseudoleucocytosis. *Thromb Haemost* 1995; 73: 506–513.
53 Lombarts AJPF, Zijlstra JJ, Peters RHM, Thomasson CG, Franck PFH. Accurate platelet counting in an insidious case of pseudothrombocytopenia. *Clin Chem Lab Med* 1999; 37: 1063–1066.
54 Zucker MB, Grant RA. Nonreversible loss of platelet aggregability induced by calcium deprivation. *Blood* 1978; 52: 505–513.
55 Packham MA, Bryant NL, Guccione MA. Agglutination of rabbit platelets in plasma by the thrombin inhibitor D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone. *Thromb Haemost* 1990; 63: 282–285.
56 Zucker MB. Effect of heparin on platelet function. *Thromb Diath Haemorrh* 1975; 33: 63–65.
57 Lages B, Weiss HJ. Dependence of human platelet functional responses on divalent cations: Aggregation and secretion in heparin- and hirudin-anticoagulated platelet-rich plasma and the effects of chelating agents. *Thromb Haemost* 1981; 45: 173–179.
58 Herrmann RG, Lacefield WB, Crowe VG. Effect of ionic calcium and magnesium on human platelet aggregation. *Proc Soc Exp Biol Med* 1970; 135: 100–103.
59 Packham MA, Bryant NL, Guccione MA, Kinlough-Rathbone RL, Mustard JF. Effect of the concentration of Ca^{2+} in the suspending medium on the responses of human and rabbit platelets to aggregating agents. *Thromb Haemost* 1989; 62: 968–976.
60 Guccione MA, Packham MA, Kinlough-Rathbone RL, Perry DW, Mustard JF. Reactions of polylysine with human platelets in plasma and in suspensions of washed platelets. *Thromb Haemost* 1976; 36: 360–375.
61 Packham MA, Kinlough-Rathbone RL, Mustard JF. Thromboxane A_2 causes feedback amplification involving extensive thromboxane A_2 formation upon close contact of human platelets in media with a low concentration of ionized calcium. *Blood* 1987; 70: 647–651.
62 Cazenave J-P, Guccione MA, Packham MA, Mustard JF. Two phases of ristocetin-induced platelet aggregation. In: *Proceedings of the 16th Meeting of the American Society of Hematology* 1973: 163.
63 Phillips DR, Teng W, Arfsten A et al. Effect of Ca^{2+} on GPIIb–IIIa interactions with Integrilin. Enhanced GP IIb–IIIa binding and inhibition of platelet aggregation by reductions in the concentration of ionized calcium in plasma anticoagulated with citrate. *Circulation* 1997; 96: 1488–1494.
64 Rebello SS, Huang J, Saito K, Lucchesi BR. In vivo efficacy of SM-20302, a GP IIb/IIIa receptor antagonist, correlates with ex vivo platelet inhibition in heparinized blood but not in citrated blood. *Arterioscler Thromb Vasc Biol* 1998; 18: 954–960.
65 Storey RF, Wilcox RG, Heptinstall S. Differential effects of glycoprotein IIb/IIIa antagonists on platelet microaggregate and macroaggregate formation and effect of anticoagulant on antagonist potency: implications for assay methodology and comparison of different antagonists. *Circulation* 1998; 98: 1616–1621.
66 Leist ER, Banwell JG. Products containing aspirin. *N Engl J Med* 1974; 291: 710–712.
67 Malmsten C, Hamberg M, Svensson J, Samuelsson B. Physiological role of an endoperoxide in human platelets: hemostatic defect due to platelet cyclo-oxygenase deficiency. *Proc Natl Acad Sci USA* 1975; 72: 1446–1450.
68 Brinkhous KM, Read MS. Fixation of platelets and platelet agglutination/aggregation tests. *Methods Enzymol* 1989; 169: 149–163.
69 Davey MG, Lander H. Effect of adenosine diphosphate on circulating platelets in man. *Nature* 1964; 201: 1037–1039.
70 Born GVR, Cross MJ. The effect of adenosine diphosphate on the concentration of platelets in circulating blood. *Nature* 1963; 197: 974–976.
71 Jørgensen L, Rowsell HC, Hovig T, Glynn MF, Mustard JF. Adenosine diphosphate-induced platelet aggregation and myocardial infarction in swine. *Lab Invest* 1967; 17: 616–644.

Cambridge Books Online © Cambridge University Press, 2009

72 Holmsen I, Holmsen H. Partial purification and characterization of an ADP phosphohydrolase from human plasma. *Thromb Diath Haemorrh* 1971; 26: 177–191.

73 Lüthje J. Extracellular adenine compounds, red blood cells and haemostasis: facts and hypotheses. *Blut* 1989; 59: 367–374.

74 Marcus AJ, Broekman MJ, Drosopoulos JH et al. The endothelial cell ecto-ADPase responsible for inhibition of platelet function is CD39. *J Clin Invest* 1997; 99: 1351–1360.

75 Gaarder A, Jonsen J, Laland S, Hellem AJ, Owren PA. Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. *Nature* 1961; 192: 531–532.

76 Reimers RC, Sutera SP, Joist JH. Potentiation by red blood cells of shear-induced aggregation: Relative importance of chemical and physical mechanisms. *Blood* 1984; 64: 1200–1208.

77 Saniabadi AR, Lowe GDO, Barbenel JC, Forbes CD. A comparison of spontaneous platelet aggregation in whole blood with platelet rich plasma: additional evidence for the role of ADP. *Thromb Haemost* 1984; 51: 115–118.

78 Carter AJ, Heptinstall S. Platelet aggregation in whole blood: the role of thromboxane A_2 and adenosine diphosphate. *Thromb Haemost* 1985; 54: 612–616.

79 Cattaneo M, Canciani MT, Lecchi A et al. Released adenosine diphosphate stabilizes thrombin-induced human platelet aggregates. *Blood* 1990; 75: 1081–1088.

80 Trumel C, Payrastre B, Plantavid M et al. A key role of adenosine diphosphate in the irreversible platelet aggregation induced by the PAR1-activating peptide through the late activation of phosphoinositide 3-kinase. *Blood* 1999; 94: 4156–4165.

81 Léon C, Hechler B, Freund M et al. Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. *J Clin Invest* 1999; 104: 1731–1737.

82 Mustard JF, Packham MA, Kinlough-Rathbone RL, Perry DW, Regoeczi E. Fibrinogen and ADP-induced aggregation. *Blood* 1978; 52: 453–466.

83 Macmillan DC, Sim AK. A comparative study of platelet aggregation in man and laboratory animals. *Thromb Diath Haemorrh* 1970; 24: 385–394.

84 Meyers KM, Katz JB, Clemmons RM, Smith JB, Holmsen H. An evaluation of the arachidonate pathway of platelets from companion and food-producing animals, mink, and man. *Thromb Res* 1980; 20: 13–24.

85 Nunn B. Some characteristics of mouse platelet aggregation and a comparison of the activity of a range of compounds in mouse and human platelet-rich plasma in vitro. *Thromb Haemost* 1981; 45: 1–5.

86 O'Brien JR. Changes in platelet membranes possibly associated with platelet stickiness. *Nature* 1966; 212: 1057–1058.

87 Rozenberg MC, Holmsen H. Adenine nucleotide metabolism of blood platelets. IV. Platelet aggregation response to exogenous ATP and ADP. *Biochim Biophys Acta* 1968; 157: 280–288.

88 Packham MA, Ardlie NG, Mustard JF. The effect of adenine compounds on platelet aggregation. *Am J Physiol* 1969; 217: 1009–1017.

89 Baurand A, Eckly A, Bari N, Léon C, Hechler B, Cazenave J-P, Gachet P. Desensitization of the platelet aggregation response to ADP: differential down-regulation of the $P2Y_1$ and P2cyc receptors. *Thromb Haemost* 2000; 84: 484–491.

90 Ardlie NG, Perry DW, Packham MA, Mustard JF. Influence of apyrase on stability of suspensions of washed rabbit platelets. *Proc Soc Exp Biol Med* 1971; 136: 1021–1023.

91 Holme S, Sixma JJ, Wester J, Holmsen H. ADP-induced refractory state of platelets in vitro. II. Functional and ultra studies on gel filtered platelets. *Scand J Haematol* 1977; 18: 267–278.

92 Savi P, Nurden P, Nurden AT, Levy-Toledano S, Herbert JM. Clopidogrel: a review of its mechanism of action. *Platelets* 1998; 9: 3–4.

93 Quinn MJ, Fitzgerald DJ. Ticlopidine and clopidogrel. *Circulation* 1999; 100: 1667–1672.

94 Hechler B, Eckly A, Ohlmann P, Cazenave J-P, Gachet C. The P2Y1 receptor, necessary but not sufficient to support full ADP-induced platelet aggregation, is not the target of the drug clopidogrel. *Br J Haematol* 1998; 103: 858–866.

95 Geiger J, Brich J, Hönig-Liedl P et al. Specific impairment of human platelet $P2Y_{AC}$ ADP receptor-mediated signaling by the antiplatelet drug clopidogrel. *Arterioscler Thromb Vasc Biol* 1999; 19: 2007–2011.

96 Watson SP. Collagen receptor signaling in platelets and megakaryocytes. *Thromb Haemost* 1999; 82: 365–376.

97 Watson S, Berlanga O, Best D, Frampton J. Update on collagen receptor interactions in platelets: Is the two-state model still valid? *Platelets* 2000; 11: 252–258.

98 Kehrel B. Platelet-collagen interactions. *Semin Thromb Haemost* 1995; 21: 123–129.

99 Kinlough-Rathbone RL, Packham MA, Reimers H-J, Cazenave J-P, Mustard JF. Mechanisms of platelet shape change, aggregation, and release induced by collagen, thrombin, or A23,187. *J Lab Clin Med* 1977; 90: 707–719.

100 Packham MA, Kinlough-Rathbone RL, Reimers H-J, Scott S, Mustard JF. Mechanisms of platelet aggregation independent of adenosine diphosphate. In Silver MJ, Smith JB, Kocsis JJ, eds. *Prostaglandins in hematology*. New York: Spectrum Publications; 1977: 247–276.

101 Samuelsson B, Goldyne M, Granström E, Hamberg M, Hammarström S, Malmsten C. Prostaglandins and thromboxanes. *Annu Rev Biochem* 1978; 47: 997–1029.

102 Paul BZS, Jin J, Kunapuli SP. Molecular mechanism of thromboxane A_2-induced platelet aggregation. Essential role for $P2T_{AC}$ and α_{2A} receptors. *J Biol Chem* 1999; 274: 29108–29114.

103 Gresele P, Deckmyn H, Nenci GG, Vermylen J. Thromboxane synthase inhibitors, thromboxane receptor antagonists and dual blockers in thrombotic disorders. *Trends Pharmacol Sci* 1991; 12: 158–163.

104 Coughlin SR. Protease-activated receptors and platelet function. *Thromb Haemost* 1999; 82: 353–356.

105 Vu T-KH, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* 1991; 64: 1057–1069.

Cambridge Books Online © Cambridge University Press, 2009

106 Kinlough-Rathbone RL, Rand ML, Packham MA. Rabbit and rat platelets do not respond to thrombin receptor peptides that activate human platelets. *Blood* 1993; 82: 103–106.

107 Connolly TM, Condra C, Feng D-M et al. Species variability in platelet and other cellular responsiveness to thrombin receptor-derived peptides. *Thromb Haemost* 1994; 72: 627–633.

108 Furman MI, Liu L, Benoit SE, Becker RC, Barnard MR, Michelson AD. The cleaved peptide of the thrombin receptor is a strong platelet agonist. *Proc Natl Acad Sci USA* 1998; 17: 3082–3087.

109 Reimers H-J, Kinlough-Rathbone RL, Cazenave J-P et al. In vitro and in vivo functions of thrombin-treated platelets. *Thromb Haemost* 1976; 35: 151–166.

110 Kinlough-Rathbone RL, Perry DW, Packham MA, Mustard JF. Deaggregation of human platelets aggregated by thrombin. *Thromb Haemost* 1985; 53: 42–44.

111 Kinlough-Rathbone RL, Packham MA, Guccione MA, Richardson M, Harfenist EJ, Mustard JF. Characteristics of thrombin-degranulated human platelets: Development of a method that does not use proteolytic enzymes for deaggregation. *Thromb Haemost* 1991; 65: 403–410.

112 Kinlough-Rathbone RL, Perry DW, Guccione MA, Rand ML, Packham MA. Degranulation of human platelet by thrombin receptor peptide SFLLRN: comparison with degranulation by thrombin. *Thromb Haemost* 1993; 70: 1019–1023.

113 Michelson AD, Barnard MR, Hechtman HB et al. In vivo tracking of platelets: circulating degranulated platelets rapidly lose surface P-selectin but continue to circulate and function. *Proc Natl Acad Sci USA* 1996; 93: 11877–11882.

114 Kinlough-Rathbone RL, Perry DW. Prolonged expression of procoagulant activity of human platelets degranulated by thrombin. *Thromb Haemost* 1995; 74: 958–961.

115 Mitchell JRA, Sharp AA. Platelet clumping in vitro. *Br J Haematol* 1964; 10: 78–93.

116 Mills DCB, Roberts GCK. Effects of adrenaline on human blood platelets. *J Physiol* 1967; 193: 443–453.

117 Haslam RJ. Mechanisms of blood platelet aggregation. In Johnson SA, Seegers WH eds. *Physiology of hemostasis and thrombosis*. Springfield IL: Charles C. Thomas; 1967: 88–112.

118 Ardlie NG, Cameron HA, Garrett J. Platelet activation by circulating levels of hormones: A possible link in coronary heart disease. *Thromb Res* 1984; 36: 315–322.

119 Siltanen P. Stress, coronary disease, and coronary death. *Ann Clin Res* 1987; 19: 96–103.

120 Chignard M, Lalau Keraly C, Nunez D, Coëffier E, Benveniste J. PAF-acether and platelets. In: MacIntyre DE, Gordon JL, eds. *Platelets in biology and pathology III*. Amsterdam: Elsevier Science Publishers; 1987: 289–315.

121 Whatley RE, Zimmerman GA, Prescott SM, McIntyre TM. Platelet-activating factor and PAF-like mimetics. In: *Handbook of lipid research. Lipid second messengers* 1996; 8: 239–276.

122 Benveniste J, Jouvin E, Pirotzky E et al. Platelet-activating factor (PAF-acether): Molecular aspects of its release and pharmacological actions. *Int Arch Allergy Appl Immunol* 1981; 66 (Suppl. 1): 121–126.

123 Marcus AJ, Safier LB, Ullman HL et al. Effects of acetyl glyceryl ether phosphorylcholine on human platelet function in vitro. *Blood* 1981; 58: 1027–1031.

124 Cargill DI, Cohen DS, Van Valen RG, Klimek JJ, Levin RP. Aggregation, release and desensitization induced in platelets from five species by platelet activating factor (PAF). *Thromb Haemost* 1983; 49: 204–207.

125 Baumgartner HR, Born GVR. Effects of 5-hydroxytryptamine on platelet aggregation. *Nature* 1968; 218: 137–141.

126 Tschopp TB. Aggregation of cat platelets in vitro. *Thromb Diath Haemost* 1970; 23: 601–620.

127 de Clerck F, David JL, Janssen PA. Inhibition of 5-hydroxytryptamine-induced and -amplified human platelet aggregation by ketanserin (R 41,468), a selective 5-HT2-receptor antagonist. *Agents Actions* 1982; 12: 388–397.

128 Peters JR, Grahame-Smith DG. Human platelet 5HT receptors: characterisation and functional association. *Eur J Pharmacol* 1980; 68: 243–256.

129 de Clerck F, Xhonneux B, Leysen J, Janssen PAJ. The involvement of 5-HT_2-receptor sites in the activation of cat platelets. *Thromb Res* 1984; 33: 305–321.

130 Wun T. Vasopressin and platelets: a concise review. *Platelets* 1997; 8: 15–21.

131 Haslam RJ, Rosson GM. Aggregation of human platelets by vasopressin. *Am J Physiol* 1972; 223: 958–967.

132 Wun T, Paglieroni TG, Lachant NA. Desmopressin stimulates the expression of P-selectin on human platelets in vitro. *J Lab Clin Med* 1995; 125: 401–409.

133 Grant JA, Scrutton MC. Positive interaction between agonists in the aggregation of human blood platelets: interaction between ADP, adrenaline and vasopressin. *Br J Haematol* 1980; 44: 109–125.

134 Lachant NA, Smith MR, Xie Z-J, Romani WR, Zhou J. Heterogeneity of the aggregation response of human platelets to arginine vasopressin. *Am J Hematol* 1995; 49: 56–66.

135 Balduini CL, Noris P, Belletti S, Spedini P, Gamba G. In vitro and in vivo effects of desmopressin on platelet function. *Haematologica* 1999; 84: 891–896.

136 Wun T, Paglieroni TG, Lachant NA. Physiologic concentrations of arginine vasopressin activate human platelets in vitro. *Br J Haematol* 1996; 92: 968–972.

137 Mosher DF. Physiology of thrombospondin. *Annu Rev Med* 1990; 41: 85–97.

138 Legrand C, Dubernard V, Rabhi-Sabile S, Morandi da Silva V. Functional and clinical significance of thrombospondin. *Platelets* 1997; 8: 211–223.

139 Jaffe EA, Leung LL, Nachman RL, Levin RI, Mosher DF. Thrombospondin is the endogenous lectin of human platelets. *Nature* 1982; 295: 246–248.

140 Silverstein RL, Leung LLK, Nachman RL. Thrombospondin: A versatile multifunctional glycoprotein. *Arteriosclerosis* 1986; 6: 245–253.

141 Tuszynski GP, Rothman VL, Murphy A, Siegler K, Knudsen KA. Thrombospondin promotes platelet aggregation. *Blood* 1988; 72: 109–115.

Cambridge Books Online © Cambridge University Press, 2009

142 Parise LV. Integrin $\alpha_{IIb}\beta_3$ signaling in platelet adhesion and aggregation. *Curr Opin Cell Biol* 1999; 11: 597–601.
143 Chung J, Wand X-Q, Lindberg FP, Frazier WA. Thrombospondin-1 acts via IAP/CD47 to synergize with collagen in $\alpha 2\beta 1$-mediated platelet activation. *Blood* 1999; 94: 642–648.
144 Selak MA, Chignard M, Smith JB. Cathepsin G is a strong platelet agonist released by neutrophils. *Biochem J* 1988; 251: 293–299.
145 Rotondo S, Evangelista V, Manarini S, de Gaetano G, Cerletti C. Different requirement of intracellular calcium and protein kinase C for arachidonic acid release and serotonin secretion in cathepsin G-activated platelets. *Thromb Haemost* 1997; 78: 919–925.
146 Si-Tahar M, Renesto P, Falet H, Rendu F, Chignard M. The phospholipase C/protein kinase C pathway is involved in cathepsin G-induced human platelet activation: comparison with thrombin. *Biochem J* 1996; 313: 401–408.
147 Selak MA. Cathepsin G and thrombin: evidence for two different platelet receptors. *Biochem J* 1994; 297: 269–275.
148 Sambrano GR, Huang W, Faruqi T, Mahrus S, Craik C, Coughlin SR. Cathepsin G activates protease-activated receptor-4 in human platelets. *J Biol Chem* 2000; 275: 6819–6823.
149 Kinlough-Rathbone RL, Perry DW, Rand ML, Packham MA. Role of secreted adenosine diphosphate in the synergistic effects of cathepsin G on human platelets. *Thromb Res* 1999; 95: 341–346.
150 Kinlough-Rathbone RL, Perry DW, Rand ML, Packham MA. Effects of cathepsin G pretreatment of platelets on their subsequent responses to aggregating agents. *Thromb Res* 1999; 95: 315–323.
151 Adelman B, Michelson AD, Loscalzo J, Greenberg J, Handin RI. Plasmin effect on platelet glycoprotein IB-von Willebrand factor interactions. *Blood* 1985; 65: 32–40.
152 Pasche B, Ouimet H, Francis S, Loscalzo J. Structural changes in platelet glycoprotein IIb/IIIa by plasmin: Determinants and functional consequences. *Blood* 1994; 83: 404–414.
153 Rabhi-Sabile S, Pidard D. Exposure of human platelets to plasmin results in the expression of irreversibly active fibrinogen receptors. *Thromb Haemost* 1995; 73: 693–701.
154 Coller BS. Platelets in cardiovascular thrombosis and thrombolysis. In: Fozzard HA, Haber E, Jennings RB, Katz AM, Morgan HE, eds. *The heart and cardiovascular system* 2nd edn. New York, NY: Raven Press; 1992: 219–273.
155 Niewiarowski S, Senyi AF, Gillies P. Plasmin-induced platelet aggregation and platelet release reaction. *J Clin Invest* 1973; 52: 1647–1659.
156 Schafer AI, Maas AK, Ware JA, Johnson PC, Rittenhouse SE, Salzman EW. Platelet protein phosphorylation, elevation of cytosolic calcium, and inositol phospholipid breakdown in platelet activation induced by plasmin. *J Clin Invest* 1986; 78: 73–79.
157 Miller JL, Katz AJ, Feinstein MB. Plasmin inhibition of thrombin-induced platelet aggregation. *Thromb Diath Haemorrh* 1975; 33: 286–309.
158 Schafer AI, Adelman B. Plasmin inhibition of platelet function and of arachidonic acid metabolism. *J Clin Invest* 1985; 75: 456–461.
159 Penny WF, Ware JA. Platelet activation and subsequent inhibition by plasmin and recombinant tissue-type plasminogen activator. *Blood* 1992; 79: 91–98.
160 Lu BH, Soria C, Cramer EM et al. Temperature dependence of plasmin-induced activation or inhibition of human platelets. *Blood* 1991; 77: 996–1005.
161 Vouret-Craviari V, Grall D, Chambard J-C, Rasmussen UB, Pouysségur J, Van Obberghen-Schilling E. Post-translational and activation-dependent modifications of the G protein-coupled thrombin receptor. *J Biol Chem* 1995; 270: 8367–8372.
162 Kinlough-Rathbone RL, Perry DW, Rand ML, Packham MA. Pretreatment of human platelets with plasmin inhibits responses to thrombin, but potentiates responses to low concentrations of aggregating agents, including the thrombin receptor activating peptide, SFLLRN. *Thromb Haemost* 1997; 77: 741–747.
163 Kuliopulos A, Covic L, Seeley SK, Sheridan PJ, Helin J, Costello CE. Plasmin desensitization of the PAR1 thrombin receptor: Kinetics, sites of truncation, and implications for thrombolytic therapy. *Biochemistry* 1999; 38: 4572–4585.
164 Rabhi-Sabile S, De Romeuf C, Pidard D. On the mechanism of plasmin-induced aggregation of human platelets: Implications of secreted von Willebrand factor. *Thromb Haemost* 1998; 79: 1191–1198.
165 MacIntyre DE, Gordon JL. Calcium-dependent stimulation of platelet aggregation by PGE_2. *Nature* 1975; 258: 337–339.
166 Vezza R, Roberti R, Nenci GG, Gresele P. Prostaglandin E_2 potentiates platelet aggregation by priming protein kinase C. *Blood* 1993; 85: 2704–2713.
167 Kowalska MA, Badellino K. β-Amyloid protein induces platelet aggregation and supports platelet adhesion. *Biochem Biophys Res Commun* 1994; 205: 1829–1835.
168 Wolozin B, Maheshwari S, Jones C et al. β-Amyloid augments platelet aggregation: Reduced activity of familial angiopathy-associated mutants. *Mol Psychiat* 1998; 3: 500–507.
169 Kowalska MA, Ratajczak MZ, Majka M et al. Stromal cell-derived factor-1 and macrophage-derived chemokine: 2 chemokines that activate platelets. *Blood* 2000; 96: 50–57.
170 Oda A, Miyakawa Y, Druker BJ et al. Thrombopoietin primes human platelet aggregation induced by shear stress and by multiple agonists. *Blood* 1996; 87: 4664–4670.
171 Pasquet J-M, Gross BS, Gratacap M-P et al. Thrombopoietin potentiates collagen receptor signaling in platelets through a phosphatidylinositol 3-kinase-dependent pathway. *Blood* 2000; 95: 3429–3434.
172 Kowalska MA, Juliano D, Trybulec M, Lu W, Niewiarowski S. Zinc ions potentiate adenosine diphosphate-induced platelet aggregation by activation of protein kinase C. *J Lab Clin Med* 1994; 123: 102–109.
173 Trybulec M, Kowalska MA, McLane MA, Silver L, Lu W, Niewiarowski S. Exposure of platelet fibrinogen receptors by

Cambridge Books Online © Cambridge University Press, 2009

zinc ions: role of protein kinase C. *Proc Soc Exp Biol Med* 1993; 203: 108–116.
174 Gordon PR, Woodruff CW, Anderson HL, O'Dell BL. Effect of acute zinc deprivation on plasma zinc and platelet aggregation in adult males. *Am J Clin Nutr* 1982; 35: 113–119.
175 Henson PM, Ginsberg MH. Immunological reactions of platelets. In Gordon JL, ed. *Platelets in biology and pathology* – 2. Amsterdam: Elsevier/North Holland; 1981: 265–308.
176 Pfueller SL. Immunology of the platelet surface. In George JN, Nurden AT, Phillips DR, eds. *Platelet membrane glycoproteins*. New York: Plenum Press; 1985: 327–355.
177 Warkentin TE. Heparin-induced thrombocytopenia: a ten-year retrospective. *Annu Rev Med* 1999; 50: 129–147.
178 Newman PM, Chong BH. Heparin-induced thrombocytopenia: new evidence for the dynamic binding of purified anti-PF4-heparin antibodies to platelets and the resultant platelet activation. *Blood* 2000; 96: 182–187.
179 Hughes M, Hayward CPM, Warkentin TE, Horsewood P, Chorneyko KA, Kelton JG. Morphological analysis of microparticle generation in heparin-induced thrombocytopenia. *Blood* 2000; 96: 188–194.
180 Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes (1). *N Engl J Med* 1992; 326: 242–250.
181 Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes (2). *N Engl J Med* 1992; 326: 310–318.
182 Tangelder GJ, Slaaf DW, Arts T, Reneman RS. Wall shear rate in arterioles in vivo: Least estimates from platelet velocity profiles. *Am J Physiol* 1988; 254: H1059–H1064.
183 Chow TW, Hellums JD, Moake JL, Kroll MH. Shear-stress induced von Willebrand factor binding to platelet glycoprotein Ib initiates calcium influx associated with aggregation. *Blood* 1992; 80: 113–120.
184 Ruggeri ZM, Dent JA, Saldivar E. Contribution of distinct adhesive interactions to platelet aggregation in flowing blood. *Blood* 1999; 94: 172–178.
185 Ruggeri ZM. Old concepts and new developments in the study of platelet aggregation. *J Clin Invest* 2000; 105: 699–701.
186 Kulkarni S, Dopheide SM, Yap CL et al. A revised model of platelet aggregation. *J Clin Invest* 2000; 105: 783–791.
187 Goto S, Sakai H, Goto M et al. Enhanced shear-induced platelet aggregation in acute myocardial infarction. *Circulation* 1999; 99: 608–613.
188 Tanigawa T, Nishikawa M, Kitai T et al. Increased platelet aggregability in response to shear stress in acute myocardial infarction and its inhibition by combined therapy with aspirin and cilostazol after coronary intervention. *Am J Cardiol* 2000; 85: 1054–1059.
189 Peerschke EI, Zucker MB. Fibrinogen receptor exposure and aggregation of human blood platelets produced by ADP and chilling. *Blood* 1981; 57: 663–670.
190 Okita JR, Frojmovic MM, Kristopeit S, Wong T, Kunicki TJ. Montreal Platelet Syndrome: a defect in calcium-activated neutral proteinase (calpain). *Blood* 1989; 74: 715–721.
191 Packham MA. Methods for detection of hypersensitive platelets. *Thromb Haemost* 1978; 40: 175–195.
192 Packham MA, Kinlough-Rathbone RL. Mechanisms of atherogenesis and thrombosis. In Bloom AL, Forbes CD, Thomas DP, Tuddenham EGD, eds. *Haemostasis and thrombosis*, 3rd edn., vol. 2. Edinburgh: Churchill Livingstone; 1994: 1107–1138.
193 Trip MD, Cats VM, van Capelle FJ, Vreeken J. Platelet hyperreactivity and prognosis in survivors of myocardial infarction. *N Engl J Med* 1990; 322: 1549–1554.
194 Meade TW, Cooper JA, Miller GJ. Platelet counts and aggregation measures in the incidence of ischaemic heart disease. *Thromb Haemost* 1997; 78: 926–929.
195 Donati MB, de Gaetano G, Vermylen J. Evidence that bovine factor VIII, not bovine fibrinogen, aggregates human platelets. *Thromb Res* 1973; 2: 97–104.
196 Forbes CD, Prentice CRM. Aggregation of human platelets by purified porcine and bovine antihaemophilic factor. *Nature New Biol* 1973; 241: 149–150.
197 Hourani SM, Cusack NJ. Pharmacological receptors on blood platelets. *Pharmacol Rev* 1991; 43: 243–298.
198 Gresele P, Arnout J, Deckmyn H, Vermylen J. Mechanism of the antiplatelet action of dipyridamole in whole blood: modulation of adenosine concentration and activity. *Thromb Haemost* 1986; 55: 12–18.
199 Moncada S, Vane JR. Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A_2, and prostacyclin. *Pharmacol Rev* 1978; 30: 293–331.
200 Nishizawa EE, Miller WL, Gorman RR, Bundy GL, Svensson J, Hamberg M. Prostaglandin D_2 as a potential antithrombotic agent. *Prostaglandins* 1975; 9: 109–121.
201 Moncada S, Palmer RM, Higgs EA. Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991; 43: 109–142.
202 Radomski MW, Palmer RM, Moncada S. Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets. *Br J Pharmacol* 1987; 92: 181–187.
203 Emerson M, Momi S, Paul W, Alberti PF, Page C, Gresele P. Endogenous nitric oxide acts as a natural antithrombotic agent *in vivo* by inhibiting platelet aggregation in the pulmonary vasculature. *Thromb Haemost* 1999; 81: 961–966.
204 Mills DCB. ADP receptors on platelets. *Thromb Haemost* 1996; 76: 835–856.
205 Blajchman MA. An overview of the mechanism of action of antithrombin and its inherited deficiency states. *Blood Coag Fibrinolysis* 1994; 5 (Suppl. 1): S5–S11.
206 van't Veer C, Mann KG. Regulation of tissue factor initiated thrombin generation by the stoichiometric inhibitors tissue factor pathway inhibitor, antithrombin-III, and heparin cofactor-II. *J Biol Chem* 1997; 272: 4367–4377.
207 Esmon CT, Ding W, Yasuhiro K et al. The protein C pathway: new insights. *Thromb Haemost* 1997; 78: 70–74.
208 Mustard JF, Packham MA. Factors influencing platelet function: Adhesion, release, and aggregation. *Pharmacol Rev* 1970; 22: 99–187.

Cambridge Books Online © Cambridge University Press, 2009

209 White JG, Rao GHR, Gerrard JM. Effects of the ionophore A23187 on blood platelets. *Am J Pathol* 1974; 77: 135–149.
210 Zucker MB, Troll W, Belman S. The tumor-promoter phorbol ester (12-o-tetradecanoyl-phorbol-13-acetate), a potent aggregating agent for blood platelets. *J Cell Biol* 1974; 60: 325–336.
211 Kajikawa N, Kikkawa U, Itoh K, Nishizuka Y. Membrane permeable diacylglycerol, its application to platelet secretion and regulation of platelet protein kinase C. *Methods Enzymol* 1989; 169: 430–442.
212 Packham MA, Livne A-A, Ruben DH, Rand ML. Activation of phospholipase C and protein kinase C has little involvement in ADP-induced primary aggregation of human platelets: effects of diacylglycerols, the diacylglycerol kinase inhibitor R59022, staurosporine and okadaic acid. *Biochem J* 1993; 290: 849–856.
213 Kienast J, Arnout J, Pfliegler G, Deckmyn H, Hoet B, Vermylen J. Sodium fluoride mimics effects of both agonists and antagonists on intact human platelets by simultaneous modulation of phospholipase C and adenylate cyclase activity. *Blood* 1987; 69: 859–866.
214 Stasi M, Gresele P, Porcellati S, Quero E, Nenci GG, Goracci G. Activation of phospholipase A_2 and beta-thromboglobulin release in human platelets: comparative effects of thrombin and fluoroaluminate stimulation. *Biochim Biophys Acta* 1992; 1124: 279–287.
215 Nucciarelli F, Gresele P, Nardicchi V et al. Evidence that cytosolic phospholipase A_2 is down-regulated by protein kinase C in intact human platelets stimulated with fluoroaluminate. *FEBS Lett* 1999; 450: 39–43.
216 Triplett DA. Coagulation and bleeding disorders: review and update. *Clin Chem* 2000; 46: 1260–1269.
217 Rubin R, Rand ML. Alcohol and platelet function. Alcoholism. *Clin Exp Res* 1994; 18: 105–110.
218 Kannel WB, Ellison RC. Alcohol and coronary heart disease: the evidence for a protective effect. *Clin Chim Acta* 1996; 246: 59–76.

Cambridge Books Online © Cambridge University Press, 2009

24

Amplification loops: release reaction[1]

Marc F. Hoylaerts

Center for Molecular and Vascular Biology, University of Leuven, Belgium

Introduction

As a consequence of vessel wall damage, circulating platelets adhere to subendothelial ligands, targeting them to the site of injury. Adhering platelets undergo a multitude of signalling events, which result in platelet activation and spreading. Whereas platelet spreading on collagen and binding of adhesive proteins facilitate subsequent recruitment of additional platelets from the circulation, the activation of platelets also leads to the controlled release of their contents. Thus, the local secretion of further agonists of platelet activation, via a signalling controlled exocytosis reaction, contributes to the rapid recruitment of circulating platelets to the injury site; secreted proteins also facilitate fibrin formation.

In a forming platelet aggregate, as a result of inside-out signalling, the platelet receptor for fibrinogen, i.e. GPIIb/IIIa, is assembled[1]. The binding of fibrinogen to this receptor not only enables platelet cross-linking, but further contributes to the ongoing platelet activation via outside–in signalling[2]. This highly localized enhancement of ongoing platelet activation leads to marked degranulation in a confined area. Thus, platelet aggregate formation occurs as the result of both platelet adhesion and localized secretion, leading to the formation of a platelet plug, in which large numbers of platelets are completely degranulated[3].

Platelet granules and exocytosis

Morphologically, three types of platelet granules can be released: dense core granules (δ-granules), α-granules and platelet lysosomes (λ-granules).

Platelet granule contents

Alpha-granules contain proteins relevant for platelet interactions (the GPIIb/IIIa fibrinogen receptor, fibrinogen, von Willebrand factor, thrombospondin-1, fibronectin, vitronectin, GAS6, see further), but also proteins relevant for coagulation and fibrinolysis (multimerin carrier protein for coagulation factor V, coagulation factor V, plasminogen, α_2-antiplasmin, plasminogen activator inhibitor-1, protein S), markers of platelet activation (P-selectin, β-thromboglobulin, platelet factor 4), activators of chemotaxis and cell function (transforming growth factor-β, platelet-derived growth factor) and plasma proteins (albumin, von Willebrand antigen II, immunoglobulins, plasma protease inhibitors, histidine-rich glycoprotein). Some proteins are synthesized by the megakaryocyte and platelet[4] (and references therein), others are taken up by a process of receptor mediated endocytosis. Dense granules on the contrary store serotonin, ATP, ADP, calcium and pyrophosphate; they can be distinguished morphologically from alpha-granules using electron microscopy, as detailed in Chapter 4. Finally, lysosomal granules contain enzymes such as cathepsin D, cathepsin E, carboxypeptidase A and B, proline carboxypeptidase, β-*N*-acetyl-D-hexaminidase, β-D-glucuronidase, β-D-galactosidase, α-D-mannosidase, α-L-arabinofuranosidase, α-D-galactosidase, α-L-fucosidase, β-D-fucosidase, β-D-glucosidase, α-D-glucosidase, acid phosphatase and arylsulphatase[5].

Lysosomes (λ-granules) differ from the other platelet granules by the type of stimulus required to induce secretion. Indeed, while the release of α- or δ-granules can be achieved by stimulation with almost all known agonists, including weak stimuli such as ADP, the secretion of λ-granules requires strong stimuli, such as thrombin or high doses of collagen[6,7]. Much less attention has been given to

[1] To the memory of my father

the molecular pathways regulating lysosomal exocytosis in platelets than to those controlling α- and δ-granule secretion. Nevertheless, it was recently shown that λ-granule release takes place in vivo, and that markers of lysosomal secretion can be detected in the blood oozing from a skin wound inflicted for the measurement of the bleeding time[8].

Characteristics of platelet dense granules

Although the lumen of platelet dense granules is less acidic than that of conventional lysosomes and appears to be devoid of lysosomal hydrolases, there is accumulating evidence that these organelles belong to the lysosomal lineage. The dense granule membrane appears to be enriched in the lysosomal proteins CD63/LAMP-3 and LAMP-2[9]. Moreover, genetic disorders that affect the biogenesis of melanosomes and lysosomes also result in platelet dense granule deficiency[10]. Further, a H^+ pumping ATPase, distinct from that at the plasma membrane, acts to maintain the low pH of the granule lumen[11]. Low molecular mass monomeric GTP binding proteins in platelets[12] are involved in the docking of granules to the plasma membrane, preceding exocytosis (see further: GTP-binding proteins in exocytosis). Dense granule membranes contain typical plasma membrane receptor complexes such as GPIb and GPIIb/IIIa, as revealed by immunohistochemical studies[13]. Dense granules also contain P-selectin, a leukocyte binding protein, initially localized in α-granules[14].

Dense granules, like α-granules, arise from both endogenous synthesis in the megakaryocyte as well as from heterotypic fusion with endocytic vesicles budding from the plasma membrane[13].

Physiology of dense granule exocytosis

Intragranular concentrations were calculated to be 65 mM for serotonin, 436 mM for ATP, 653 mM for ADP, 2.2 M for calcium and 326 mM for pyrophosphate[5]. The granular adenine nucleotide pool, which is likely in the form of insoluble calcium complexes, is distinct from the cytoplasmic nucleotides, and these two pools are not readily exchangeable. Serotonin is not synthesized in megakaryocytes/platelets but is actively taken up from the plasma and accumulated in dense granules, where it is likely complexed with ATP and potentially with calcium. The serotonin released by exocytosis is relatively stable and functions as a weak platelet agonist on $5HT_2$ receptors[15]. ADP released during exocytosis is capable of interacting with two different receptors on the platelet $P2Y_1$, that activates phospholipase C via $Gq\alpha$[16] and $P2Y_{12}$, that inhibits adenyl cyclase via $Gi\alpha$[17]. ADP has been shown to play a central role in secondary platelet aggregation following induction of platelet activation by various agonists such as thrombin, TXA_2 and collagen[18,19]. Released ADP can also interact with P2Y receptors present on endothelial cells to release nitric oxide and prostacyclin, which in turn cause vasodilation[20]. Dense granules also release ATP; platelets contain a membrane receptor for ATP, called $P2X_1$, which is a ligand gated ion-channel, causing extremely fast inwardly rectifying Ca^{2+}-currents upon stimulation with ATP. The role of this ion-channel in blood platelet activation however still is unclear[21]. We have recently described a mutation of the $P2X_1$ gene and a defective $P2X_1$ ion channel in a patient with a bleeding disorder, suggesting a role for platelet $P2X_1$ in haemostasis[22]. At physiological Ca^{2+}-concentrations, stimulation of the $P2X_1$ ion channel triggers fast and reversible platelet shape change and causes protein kinase C activation[23]. Fig. 24.1 summarizes how dense granule release amplifies platelet aggregation. Lages and Weiss[24] have also emphasized that secreted dense granule adenine nucleotides promote calcium influx and the maintenance of elevated cytosolic calcium levels in stimulated human platelets, thereby sustaining secondary platelet aggregation.

α-granule release

As outlined above, during exocytosis, platelet α-granules release several proteins into the surrounding medium or onto the surface of the platelet, which participate in platelet aggregate formation and stabilization. Thus, the large von Willebrand factor multimers secreted by platelets play a role in the initial platelet aggregate formation in flowing blood; they interact with both the GPIb complex and the GPIIb/IIIa receptor on platelets streaming by and activate platelets via these receptors[25]; these interactions are reinforced by the binding of fibrinogen to GPIIb/IIIa. Released thrombospondin-1 stabilizes aggregates by interacting with GPIIb/IIIa bound fibrinogen[26]. The α-granule membrane protein P-selectin appears on the surface of the activated platelet and mediates platelet–leukocyte interaction via binding to its ligand on leukocytes and endothelial cells, the P-selectin glycoprotein ligand-1 (PSGL-1); this is a mucin-like transmembrane protein constitutively expressed on leukocytes[27]. Decoration by activated platelets enhances monocyte deposition onto endothelial cells[28,29]. The platelet–monocyte interaction also decrypts monocyte tissue factor by providing the phospholipid environment required for effective coagulation[30]; secreted factor V facilitates local thrombin generation. The fibrin formed on the activated platelets is stabilized by the

Cambridge Books Online © Cambridge University Press, 2009

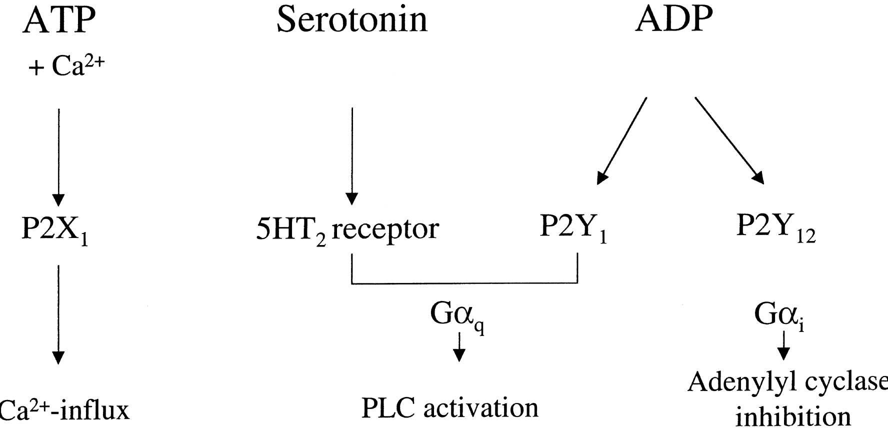


Fig. 24.1. Platelet receptor activation by dense granule release products. Dense granules release ADP, which stimulates two different G-protein coupled receptors, leading to Ca^{2+}-mobilization and to a reduction of intracellular cAMP levels. Serotonin is a weak agonist of Ca^{2+}-mobilization and released ATP will trigger fast influx of Ca^{2+}, thus modulating ongoing platelet activation.

release of inhibitors of fibrinolysis by the platelet α-granule, such as active plasminogen activator inhibitor-1 (PAI-1)[31]. Released growth factors play an important role in eliciting smooth muscle cell proliferation following vascular trauma; induction of thrombocytopenia in animal models induces a clear drop in medial smooth muscle cell migration and proliferation following vessel wall injury[32].

Membranes of the alpha-granules contain proteins found on the plasma membrane (e.g. GPIIb/IIIa, CD36, CD9, Rap1b). As much as 10% of the glycoprotein Ib complex can be found associated with the alpha granule membrane[33]. Thus, the α-granule seems to develop from the homotypic fusion of trans-Golgi vesicles in megakaryocytes as well as from heterotypic fusion of these vesicles with endocytic vesicles[34].

Although dense granule release is more directly coupled to immediate amplification of platelet activation, α-granule exocytosis also contributes to platelet stimulation. Besides activation through secreted vWF, we recently found that the protein S homologous protein GAS6 is rapidly released upon platelet stimulation and binds to GAS6 receptors on the platelet surface, resulting in further platelet activation and exocytosis[35]. Fig. 24.2 schematically shows how, following a primary platelet activation signal, α- and dense granule release products promote further platelet activation and aggregation. Thus, platelet activation and release reactions have to be viewed as fast but

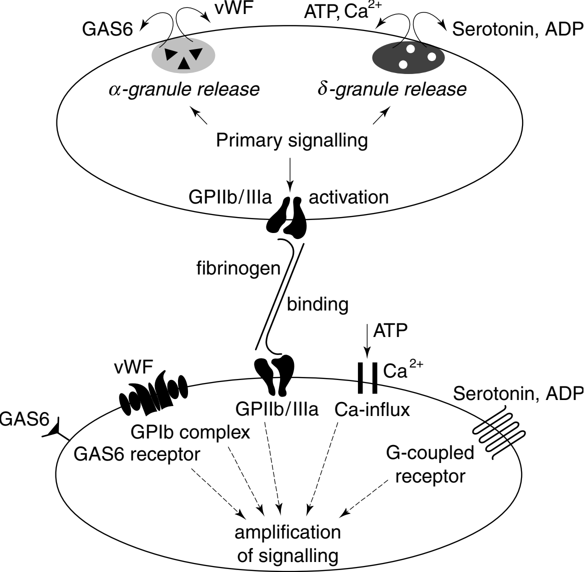


Fig. 24.2. Platelet secretion and activation are intertwined processes. Primary activation by various agonists induces intracellular signalling, leading to the release of α- and δ-granule contents (top). Released proteins from α-granules and released low molecular weight substances from δ-granules lead to further receptor mediated signalling, reinforcing ongoing activation, recruiting additional platelets and eventually leading to complete degranulation and platelet aggregation (bottom).

Cambridge Books Online © Cambridge University Press, 2009

intertwined processes, leading to the typical progressive morphological changes in platelets and to aggregation.

Formation of microvesicles

Strong platelet activation or potent shear stress not only induce granule secretion, but also the release of microvesicles[36]. Platelet-derived microvesicles have been observed in standardized bleeding time wounds[37] and have been associated with autoimmune thrombocytopenic purpura[38] and cardiopulmonary bypass[39]. Microvesicles can be analysed by flow cytometry and express at their membrane surface a number of platelet glycoproteins such as the GPIb complex, the fibrinogen receptor GPIIb/IIIa, but also P-selectin. Platelet microvesicles are fragments of the platelet membrane, released following disruption of the underlying cytoskeleton. The accumulation of phosphatidylserine on the outer membrane provides a negatively charged phospholipid surface for the binding of the γ-carboxyglutamic acid containing coagulation factors, and of factor V and factor VIII through their C_2 domain[40]. Thus, detached microvesicles have the potential to provide a procoagulant surface, also distant from the site of primary platelet activation.

In addition, vesicles can originate from exocytosis of endocytic multivesicular bodies as so-called exosomes[41]. This phenomenon also exists in platelets[42]. Therefore, during platelet activation, two different vesicle populations are released: microvesicles derived from the plasma membrane and a separate population of exosomes. The exosomes, too small to be detected via flow cytometry, are selectively enriched in the tetraspan protein CD63. Annexin V primarily binds to microvesicles and only to a small extent to exosomes. It therefore seems that released platelet exosomes have an extracellular function other than a procoagulant activity, mainly attributed to microvesicles[36].

Protein kinase C and platelet secretion

Protein kinase C activation

Several agonists of platelet activation, such as thrombin, ADP, platelet-activating factor (PAF), and thromboxane A_2 bind to a specific receptor on the platelet surface, which triggers activation of phospholipase C. This enzyme in turn catalyzes the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP_2) into 1,4,5-inositol-triphosphate (IP_3) and diacylglycerol (DG). IP_3 then mobilizes Ca^{2+} from internal stores and DG activates protein kinase C (PKC), which translocates away from the membrane and phosphorylates its substrates, the 40–47 kD protein pleckstrin and the 20 kD myosin light chain[43]. Several PKC isoenzymes have been identified, ranging from the conventional calcium-dependent cPKC (α, βI-IV and γ), the calcium-independent isoenzymes nPKC (δ, ε, η and θ) and the atypical aPKC (ζ, λ, ι and μ;[44] and ref therein). In addition to the stimulation of PKC activity in platelets by thrombin, PAF or collagen, phorbol esters such as phorbol 12-myristate 13-acetate (PMA) are capable of directly activating PKC and of inducing protein phosphorylation and platelet secretion[44] (and ref therein). Thus, platelet activation both by thrombin and by PMA is accompanied by translocation of PKC isoenzymes α, β and ζ, whereas the δ isoenzyme is specifically translocated during thrombin activation[45].

Studies focusing on the relationship between platelet secretion and PKC-dependent and tyrosine kinase-dependent phosphorylations of proteins, have claimed that protein kinase C-dependent pleckstrin phosphorylation is a prerequisite for platelet secretion. This process appears to be regulated by other phosphoproteins; in particular the serine/threonine phosphorylation of 27 and 68 kD proteins and the tyrosine phosphorylation of the p60c-src were found to be associated with a decrease in the secretory activity[46].

PKC phosphorylates MARCKS

Activation of PKC in other secretory systems, such as the chromaffin system, potentiates secretion and this effect of PKC activation is due to disassembly or disruption of cortical actin networks[47,48]. This allows a large number of secretory vesicles to move to release sites on the plasma membrane[5] (see further: cytoskeletal fibres and secretion). Elzagallaai et al.[49] hypothesized that PKC activation in platelets also produces actin filament disassembly and thereby enhances the secretory response. These authors observed that myristoylated alanine-rich C kinase substrate (MARCKS), in addition to pleckstrin, is phosphorylated during platelet activation. MARCKS has the property of binding actin and of crosslinking actin filamants[50,51]. Phosphorylation of MARCKS by PKC inhibits its ability to crosslink actin filaments. Elzagallaai et al.[49] demonstrated MARCKS phosphorylation in platelets in response to PMA stimulation. They furthermore showed that a peptide (MPSD) with the amino acid sequence corresponding to the phosphorylation site of MARCKS blocks both MARCKS phosphorylation and serotonin release from permeabilized platelets in response to PMA stimulation. Because pleckstrin and myosin light chain phosphorylation were

Cambridge Books Online © Cambridge University Press, 2009

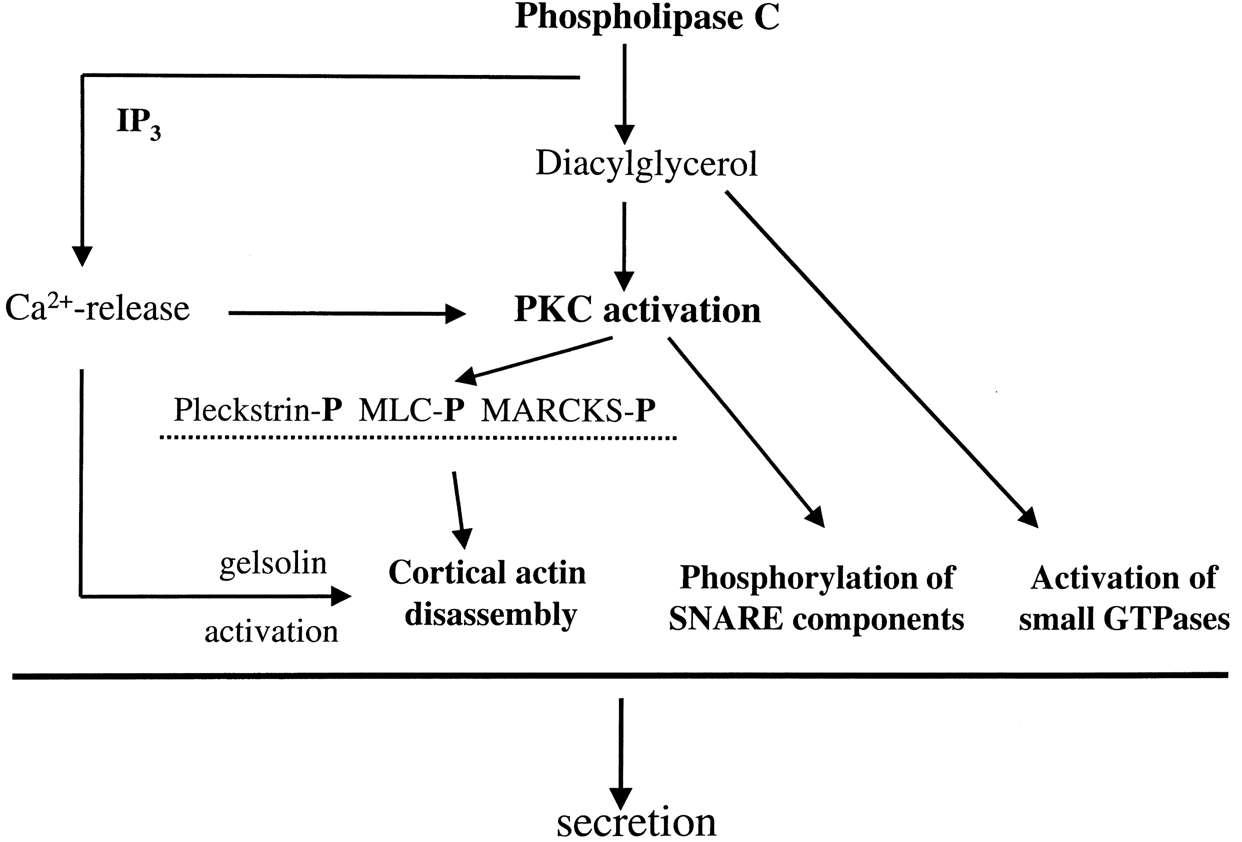


Fig. 24.3. Role of protein kinase C (PKC) in platelet exocytosis. Following receptor mediated activation of phospholipase C, diacylglycerol and Ca^{2+} activate PKC, that phosphorylates several proteins directly involved in actin disassembly. This process occurs in conjunction with PKC dependent phosphorylation of SNARE components and PKC-like activation of small GTPases, ultimately leading to secretion. MLC: myosin light chain; MARCKS: myristoylated alanine-rich C kinase substrate; PKC: protein kinase C; SNARE: SNAP receptor (see section on The SNARE family).

not affected by the peptide, the authors conclude that MARCKS may play a role in platelet secretion. Moreover, because pleckstrin phosphorylation has a different time course than that of MARCKS, they question the role of pleckstrin in secretion and suggest that it might only be involved upstream in the cascade of events leading to exocytosis. The role of MARCKS in platelet secretion is schematically represented in Fig. 24.3.

Additional roles of PKC in platelet secretion

As will be discussed on p. 362, PKC also is involved in the fusion of granules with the plasma membrane. Syntaxin 4 and a platelet sec I protein (PSP) are phosphorylated by PKC, thereby modifying the plasma membrane in a way to allow granule docking and fusion.

Furthermore, as presented on p. 364, protein kinase C-related activities may be implicated in the activation of small GTPases involved in secretion, by promoting the exchange of GDP for GTP.

PKC independent secretion

Hashimoto et al.[52] have proposed a protein kinase C-independent mechanism of dense granule exocytosis in human platelets. In agreement with this, Sloan and Haslam[53] found that secretion stimulated by Ca^{2+} or by guanosine 5–[γ-thio]-triphosphate (GTPγ[S]) and Ca^{2+} utilizes membrane-associated Ca^{2+}- and GTP-binding proteins and occurs independently of PKC activation. GTPγ[S] did not stimulate secretion from streptolysin O-permeabilized platelets in the absence of Ca^{2+}, but greatly potentiated secretion in the presence of low PMA or low Ca^{2+}. However, GTPγ[S] did stimulate myosin P-light chain phosphorylation in the absence of Ca^{2+}, an effect that was associated with morphological changes,

Cambridge Books Online © Cambridge University Press, 2009

including granule centralization. Inhibition of PKC and of pleckstrin phosphorylation by Ro 31–8220 blocked secretion induced by PMA or by GTPγ[S] and PMA in the absence of Ca^{2+}, but did not prevent the GTPγ[S]-induced phosphorylation of myosin P-light chains or secretion induced by Ca^{2+} at pCa 5. These experiments indicate that myosin light chain phosphorylation can occur independently of PKC activity and suggest that GTP-binding proteins and Ca^{2+} may induce exocytosis by activating processes downstream of PKC, as will be further discussed on p. 364 and is illustrated in Fig. 24.3.

Cytoskeletal fibres and secretion

Platelets contain at least three systems of cytoskeletal fibres: the membrane skeleton, microtubules, and microfilaments (long actin filaments). Filamentous actin rapidly increases during platelet activation[54,55]. The membrane skeleton is associated with the network of cytoplasmic actin filaments, various actin-binding proteins, and surface membrane glycoproteins[56,57]. This skeleton is a continuous layer and is preserved as such following platelet lysis with Triton X-100[58]. Circumferential bundles of microtubules play an important role in maintaining the discoid shape of resting platelets, which is lost during microtubule depolymerization[59].

Microtubules seem to be involved in platelet secretion because colchicine (a tubulin ligand[60]) and monoclonal antibodies to alpha and beta subunits of tubulin[61] inhibit secretion in permeabilized platelets. Microtubule-associated proteins may play a role in microtubule reorganization, because they regulate the stability and phosphorylation of the microtubules[62]. The contractile ring and stress fibers of platelets may also be involved in exocytosis and retraction[63]. Cytoskeletal reorganization is mediated through actin polymerization and depolymerization and is regulated by phosphatidylinositol-4,5-bisphospate (PIP_2), which binds to actin regulatory proteins such as scinderin and gelsolin[64]. Consistent with these observations, recombinant scinderin potentiates Ca^{2+}-induced serotonin release in permeabilized platelets, whereas PIP_2, or 2 peptides that interfere with scinderin-actin interactions, block exocytosis[65].

Granule and plasma membrane fusion

Recent insights into molecular mechanisms of secretion indicate that platelet granule secretion is homologous to exocytosis in neurons and other cells but involves a platelet-selective machinery that is uniquely coupled to cell activation. It is generally believed that neuronal exocytosis proceeds through a sequence of vesicle *docking*, *priming*, *triggering* and *fusion*, leading to release of neurotransmitters into the synaptic cleft[4]. The docking or close apposition of vesicles to the plasma membrane at the active site of neurons has been demonstrated by electron microscopy. However, only a subset of these vesicles will be released rapidly, indicating that additional priming or activation of the docked vesicle is necessary, a process apparently involving Mg^{2+} and ATP[66]. Increases in intracellular Ca^{2+}-concentration then trigger the release, which is mediated via SNARE complex interactions (see below).

While neurotransmitters are released from small synaptic vesicles within 200 μs, platelet granules release their contents over an interval of 2 to 5 s. Whereas in each case, exocytosis is triggered by an increase in intracellular Ca^{2+}, significant differences exist between these cells in the magnitude of the Ca^{2+}-signal and the pathway followed to generate it. In neurons, membrane depolarization down the axis elicits Ca^{2+}-influx via Ca^{2+}-channels. This leads to local Ca^{2+}-concentrations as high as 200 μmol/l, triggering the exocytosis of vesicles in close proximity. Platelet secretion on the contrary is triggered by extracellular agonists that bind to platelet receptors, coupled to G-proteins. Following activation, Ca^{2+} is liberated from internal stores and reaches intracellular concentrations ranging from 2–10 μmol/l, in conjunction with diacylglycerol formation leading to protein kinase C activation, as outlined above. The degree of exocytosis is determined by the magnitude of the activation signal and strong platelet agonists can induce full platelet degranulation. In contrast, vesicles in neurons are released in a quantal fashion[4].

The fusion process itself can be described by the general SNARE hypothesis. This hypothesis states that vesicle fusion requires the involvement of three different components: t-SNAREs, v-SNAREs and a soluble SNAP/NSF component. This hypothesis and the molecular entities involved are explained below.

The SNARE family

SNAREs (Soluble NSF Attachment protein Receptor) comprise distinct families of conserved membrane-associated proteins, which facilitate membrane fusion in eukaryotes[67]. They are found throughout the secretory pathway and participate in a number of membrane-trafficking events, including trafficking of cargo-containing carrier vesicles, compartmental organization and organelle fusion. The term SNARE refers to entities which participate in the binding of soluble factors, such as the ATPase NSF

Cambridge Books Online © Cambridge University Press, 2009

(N-ethylmaleimide-Sensitive Fusion protein) and its Soluble NSF-Attachment Proteins (SNAPs) to membranes derived from bovine brain homogenates[68,69]. The so-called SNAP Receptors (hence the name SNAREs) turned out to be proteins previously identified and characterized, at least in part, by groups studying neuronal transmission or protein trafficking in yeast.

Several reviews have outlined the molecular entities involved in SNARE mediated vesicle docking[70,71]. These molecular entities belong to the three major SNARE families: the *syntaxin*, *SNAP-25* and *synaptobrevin/VAMP* families. Two distinct categories of SNAREs have been described. SNAREs present on the vesicle compartment are known as v-SNAREs, while those on the target compartment are known as t-SNAREs. In the original study by Söllner et al.[72], the v-SNARE identified was a synaptic Vesicle-Associated Membrane Protein (abbreviated VAMP) or termed synaptobrevin. The v-SNARE family comprises the following components: *VAMP1, VAMP2, VAMP3, VAMP5, VAMP7/TI-VAMP, VAMP8/Endobrevin, Synaptotagmin I, II, III, V, X.* The t-SNARE family consists of *Syntaxin 1A* and *1B, Syntaxin 2, Syntaxin 3, Syntaxin 4, SNAP 25 A* and *B, SNAP23/Syndet.*

v- and t-SNARE regulation

The assembly of the key players in the SNARE hypothesis is schematically represented in Fig. 24.4. At the level of the presynaptic membrane in neurons, the physical association of the v-SNARE synaptobrevin (or VAMP) with both the t-SNAREs of the plasma membrane, SNAP-25 and syntaxin, leads to the formation of a stable ternary (or 'core') SNARE complex of 7S[72], that docks the vesicle to its target membrane. This core complex then binds SNAPs followed by NSF to form a particle of 20S. The central element in the SNARE hypothesis states that the specificity of membrane targeting thus relies on specific pairing of 3 SNAREs. Within the core complex, the v- and t-SNAREs are aligned in parallel[73–75]. The individual SNAREs were found to undergo a significant increase in helicity upon association with their SNARE partners[76–78], suggesting that formation of the complex is energetically favourable. This lends credence to the idea that SNARE assembly overcomes the electrostatic forces, which prevent membrane fusions from occurring. At the end of a fusion event, the highly conserved NSF, together with SNAPs, dissociates the stable v/t-SNARE complexes by using energy provided by the hydrolysis of ATP. Following this dissociation step, the vesicle and target membrane can fuse, since they have been brought into sufficiently close contact.

In addition to the key proteins in the SNARE hypothesis,

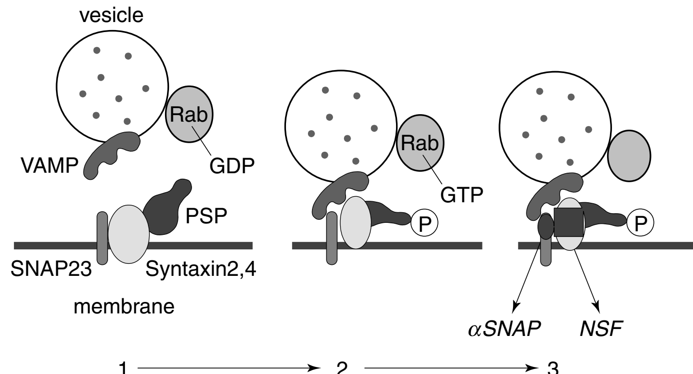


Fig. 24.4. The SNARE hypothesis. In resting platelets, membrane t-SNARES SNAP23 and syntaxin 2 and 4 are complexed. The association with the platelet secI protein PSP prevents syntaxins from interacting with the counterpart v-SNARE VAMP, present on the membrane of the vesicle (step 1). During platelet activation, phosphorylation reactions of syntaxin and of PSP weaken the interactions between SNAP23 and syntaxins and relieve the inhibition of the interaction with the v-SNARE; Rab exchanges GDP for GTP and facilitates SNARE complex formation (step 2). Thus a 3 SNARE protein complex is formed which subsequently binds the soluble proteins αSNAP and the ATPase NSF (step 3). Following ATP-hydrolysis by this enzyme, the SNARE complex disassembles, enabling the fusion of the vesicular and target membranes, which are now in physical proximity.

other factors exist that control exocytosis[67]. Thus, the Sec1/Munc 18 proteins bind to syntaxins and through this binding prevent formation of the core SNARE complex. Protein kinase C has been implicated in modulating synaptic transmission. This stems in part from studies which show that neurotransmitter release from presynaptic terminals and various other exocytic events are modulated by exposure to phorbol esters[79,80] which are known PKC agonists. Moreover, PKC has been shown to phosphorylate substrates involved in vesicle fusion (i.e. Sec1/Munc18, which thereby loses its inhibitory role on membrane fusion)[81].

SNAREs in platelets

Recent studies have revealed that the secretory machinery in platelets has important homologies to the machinery found in neurons and other cells. Platelets can form core SNARE complexes in vitro that support αSNAP-dependent NSF-ATPase activity[82] and the αSNAP-dependent NSF-ATPase activity has been shown to be critical for exocytosis of both α- and dense granules[83,84]. Platelet membranes contain the t-SNARES syntaxins 2 and 4, which play distinctive roles in granule exocytosis; syntaxin 2 is involved

Cambridge Books Online © Cambridge University Press, 2009

in dense granule release whereas syntaxin 4 is necessary for platelet α-granule secretion[82,85–87]. Platelets contain abundant amounts of the t-SNARE SNAP-23, and the v-SNARE VAMP, which interact and form SNARE complexes. SNAP-23 is required for dense granule exocytosis, but it is not yet clear which VAMP is functionally involved in secretion[84,88].

In resting secretory cells neighbouring membrane SNARE proteins are interacting with one another, thus preventing v- and t-SNAREs from fusing. Thus, Flaumenhaft et al.[86] demonstrated that the t-SNARES SNAP-23 and syntaxin 4 form a heterotrimeric complex in platelets. When human platelets were activated with a potent agonist such as thrombin, Chung et al.[89] found that syntaxin 4 was phosphorylated by PKC in human platelets. Cellular activation by thrombin or phorbol 12-myristate 13-acetate decreased the binding of syntaxin 4 to SNAP-23. A PKC inhibitor blocked syntaxin 4 phosphorylation and restored binding of syntaxin 4 to SNAP-23 to that seen in non-stimulated platelets. Thus, these experiments support a model in which phosphorylation of SNARE proteins weakens their interaction in the plasma membrane and prepares them for v-SNARE–t-SNARE interaction.

Chen et al.[90] have furthermore shown that also release from the platelet lysosome requires ATP and NSF. The SNAREs syntaxin 2 and 4, together with SNAP-23, appear to make up the heterodimeric t-SNAREs required for lysosome exocytosis.

A platelet secI protein (PSP) has been cloned, which is orthologous to Munc-18[87]. PSP forms a tight complex with syntaxin 2 and syntaxin 4 that can prevent the formation of the SNARE complex[87,91]. PSP is phosphorylated in platelets activated by thrombin through a protein kinase C-dependent mechanism; this phosphorylation weakens interaction with syntaxin 4 and relieves the inhibitory effect of PSP on SNARE complex formation. Thus, the PKC dependent phosphorylations of several proteins seem to control SNARE protein interactions.

In summary, it is clear that all three platelet exocytosis events, i.e. release from α-granules, from δ-granules and from lysosomes require the general membrane fusion protein N-ethylmaleimide Sensitive Factor (NSF)[83,84]. The role for the t-SNAREs syntaxin 2 and SNAP-23 has been established for dense granule and lysosome release[90] and syntaxin 4 appears to be required for lysosome and α-granule release[86,92].

GTP-binding proteins in exocytosis

Presence on secretory vesicles

As outlined above, exocytosis is a regulated process involving the docking of secretory granules at specific sites on the plasma membrane, with subsequent fusion and release of granule contents. In addition to the proteins involved in the SNARE hypothesis, our understanding of other proteins involved in vesicular trafficking has increased dramatically. In an extensive review paper, Watson[93] presents evidence from a genetic, biochemical, immunological and functional angle to support a role for ras-like monomeric GTP-binding proteins as well as heterotrimeric GTP-binding protein (G-protein) subunits in various steps of the vesicular trafficking pathway, including the transport of secretory vesicles to the plasma membrane.

Monomeric GTP-binding proteins may be divided into four subfamilies*: ras, rho/rac, rab* and *ARF* (ADP-ribosylation factor). Rab proteins have been found to regulate defined steps of intracellular membrane trafficking, including endocytosis and exocytosis[94].

The RabGTPase family

The RabGTPase family is composed of more than 30 members and belongs to the Ras-related small GTPase superfamily[95,96]. Like other GTPases, Rab proteins have both GTP-bound active and GDP-bound inactive forms regulated by the GDP/GTP cycles. GTP is exchanged for GDP by a specific guanine nucleotide exchange protein (GEF). The active GTP-bound forms exert their function by association with effector molecules[95–97]. RabGTPases are also controlled by a unique negative regulator named Rab GDP dissociation inhibitor (GDI)[98,99]. Rab-GDI is a cytosolic protein, which forms a complex with GDP-bound Rab proteins to inhibit the GDP/GTP exchange, and extracts Rab proteins from the membrane into the cytosol. Several RabGTPases including Rab3B, -4, -6, -8 and -27 have been demonstrated to be present in platelets[100,101]. Rab4 is present on lysosomes, Rab6, Rab 8 and Rab 27B on α-granules, Rab27A on dense granules. The small GTP-ase Rap 1B has also been found in the α-granules of platelets[102].

GEFs stimulate the dissociation of the tightly bound GDP nucleotide from the small GTP-binding protein in response to upstream signals[103]. Thus, the GEF exchanging GDP for GTP Vav is activated via tyrosine phosphorylation by Syk, which stimulates its GEF activity for Rac1[104]. Recently, it was shown that the GEF protein RasGRP possesses a single C1 domain homologous to that of PKC and appears to be a high-affinity target for diacylglycerol and

Cambridge Books Online © Cambridge University Press, 2009

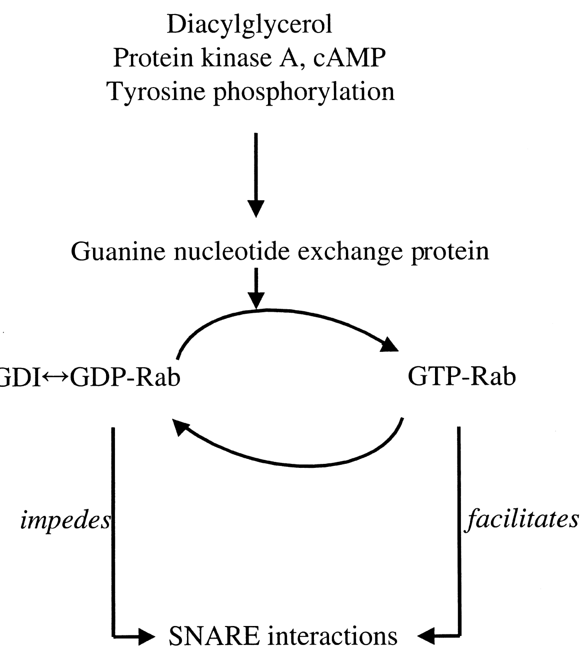


Fig. 24.5. Rab activation in SNARE interaction control. Diacylglycerol, cAMP, tyrosine phosphorylation and protein kinase A tentatively mediate activation of the guanine–nucleotide exchange protein, which causes Rab to exchange GDP for GTP. Whereas GDP-Rab impedes SNARE interactions, GTP-Rab interacts with its effector protein and promotes SNARE complex formation. Granule binding of GDP-Rab is controlled by the Rab GDP dissociation inhibitor (GDI).

phorbol esters[105], thus providing evidence of a PKC related activation process. Furthermore, the GEF for Ras CDC25Mm/GRF1 contains sites of phosphorylation for protein kinase A[106]. A guanine nucleotide exchange factor for Rap1 was described that is directly activated by cAMP[107]. One would tentatively assume that Rab-GDP, associated to the granule, impedes exocytosis, impediment that is removed by the exchange of GDP for GTP (Fig. 24.5). How this comes about requires further study.

Platelet granule dysfunctions

The importance of platelet granule secretion is illustrated by a number of clinical granule release dysfunctions that are associated with loss of platelet function and bleeding syndromes. These syndromes are discussed in more detail in Chapter 11.

Conclusion

Platelet granule exocytosis is an essential part of platelet function. Exocytosis involves the release of different granule types, all of them having their own role in platelet activation, shape change induction, adhesion and secondary amplification of ongoing platelet activation. This process is controlled via a multitude of biochemical events, ranging from receptor stimulation, intracellular signal transduction and vesicle docking and fusion. The similarity in the machinery used by the platelet and by other secretory cells makes the platelet a unique tool to study the molecular basis of vesicle-to-membrane fusion, a process that has just been initiated.

References

1 Vinogradova O, Haas T, Plow EF, Qin J. A structural basis for integrin activation by the cytoplasmic tail of the alpha IIb-subunit. *Proc Natl Acad Sci USA* 2000; 97: 1450–1455.
2 Shattil SJ. Signaling through platelet integrin alpha IIb beta 3: inside-out, outside-in, and sideways. *Thromb Haemost* 1999; 82: 318–325.
3 White JG, Krumwiede M. Further studies of the secretory pathway in thrombin-stimulated human platelet. *Blood* 1987; 69: 1196–1203.
4 Reed GL, Fitzgerald ML, Polgar J. Molecular mechanisms of platelet exocytosis: insights into the 'secrete' life of thrombocytes. *Blood* 2000; 96: 3334–3342.
5 McNicol A, Israels SJ. Platelet dense granules: structure, function and implications for haemostasis. Mini-review. *Thromb Res* 1999; 95: 1–18.
6 Holmsen H, Day HJ. The selectivity of the thrombin-induced platelet release reaction: subcellular localization of released and retained constituents. *J Lab Clin Med* 1970; 75: 840–55.
7 Metzelaar MJ, Clevers HC. Lysosomal membrane glycoproteins in platelets. *Thromb Haemost* 1992; 68: 378–382.
8 Ciferri S, Emiliani C, Guglielmini G, Orlacchio A, Nenci GG, Gresele P. Platelets release their lysosomal content in vivo in humans upon activation. *Thromb Haemost* 2000; 83: 157–164.
9 Nishibori M, Cham B, McNicol A, Shalev A, Jain N, Gerrard JM. The protein CD63 is in platelet dense granules, is deficient in a patient with Hermansky–Pudlak syndrome, and appears identical to granulophysin. *J Clin Invest* 1993; 91: 1775–1782.
10 Dell'angelica EC, Mullins C, Caplan S, Bonifacino JS. Lysosome-related organelles. *FASEB J* 2000; 14: 1265–1278.
11 Dean GE, Fishkes H, Nelson PJ, Rudnick G. The hydrogen ion-pumping adenosine triphosphatase of platelet dense granule membrane. Differences from F1F0- and phosphoenzyme-type ATPases. *J Biol Chem* 1984; 259; 9569–9574.

Cambridge Books Online © Cambridge University Press, 2009

12 Mark BL, Jilkina O, Bhullar RP. Association of Ral GTP-binding protein with human platelet dense granules. *Biochem Biophys Res Commun* 1996; 225: 40–46.

13 Youssefian T, Masse JM, Rendu F, Guichard J, Cramer EM. Platelet and megakaryocyte dense granules contain glycoproteins Ib and IIb-IIIa. *Blood* 1997; 89: 4047–4057.

14 Israels SJ, Gerrard JM, Jacques YV, McNicol A, Cham B, Nishibori M, Bainton DF. Platelet dense granule membranes contain both granulophysin and P-selectin (GMP-140). *Blood* 1992; 80: 143–152.

15 De Clerck F, Xhonneux B, Leysen J, Janssen PA. Evidence for functional 5-HT2 receptor sites on human blood platelets. *Biochem Pharmacol* 1984; 33: 2807–2811.

16 Cattaneo M, Gachet. ADP receptors and clinical bleeding disorders. *Arterioscler Thromb Vasc Biol* 1999; 19: 2281–2285. Review.

17 Hollopeter G, Jantzen H-M, Vincent D et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* 2001, 409; 202–207.

18 Storey RF, Sanderson HM, White AE, May JA, Cameron KE, Heptinstall S. The central role of the P2T receptor in amplification of human platelet activation, aggregation, secretion and procoagulant activity. *Br J Haematol* 2000; 110: 925–934.

19 Ohlmann P, Eckly A, Freund M, Cazenave JP, Offermanns S, Gachet C. ADP induces partial platelet aggregation without shape change and potentiates collagen-induced aggregation in the absence of Galphaq. *Blood* 2000; 96: 2134–2139.

20 Malmsjo M, Bergdahl A, Zhao XH et al. Enhanced acetylcholine and P2Y-receptor stimulated vascular EDHF-dilatation in congestive heart failure. *Cardiovasc Res* 1999; 43: 200–209.

21 Clifford EE, Parker K, Humphreys BD, Kertesy SB, Dubyak GR. The P2X1 receptor, an adenosine triphosphate-gated cation channel, is expressed in human platelets but not in human blood leukocytes. *Blood* 1998; 91: 3172–3181.

22 Oury C, Toth-Zsamboki E, Van Geet C et al. A natural dominant negative P2X1 receptor due to deletion of a single amino acid residue. *J Biol Chem* 2000; 275: 22611–22614.

23 Oury C, Toth-Zsamboki E, Thys C, Tytgat J, Vermylen J, Hoylaerts M.F. The $P2X_1$ ion channel in platelets acts as a functional receptor for ATP and is weakly antagonized by ADP. *Thromb Haemost* 2001; 86: 1264–1271.

24 Lages B, Weiss HJ. Secreted dense granule adenine nucleotides promote calcium influx and the maintenance of elevated cytosolic calcium levels in stimulated human platelets. *Thromb Haemost* 1999; 81: 286–292.

25 Ruggeri ZM, Dent JA, Saldivar E. Contribution of distinct adhesive interactions to platelet aggregation in flowing blood. *Blood* 1999; 94: 172–178.

26 Bonnefoy A, Hantgan R, Legrand C, Frojmovic MM. A model of platelet aggregation involving multiple interactions of thrombospondin-1, fibrinogen and GPIIbIIIa receptor. *J Biol Chem* 2000; 276: 5605–5612.

27 Moore KL. Structure and function of P-selectin glycoprotein ligand-1. *Leuk Lymphoma* 1998; 29: 1–15.

28 Theilmeier G, Lenaerts T, Remacle C, Collen D, Vermylen J, Hoylaerts MF. Circulating activated platelets assist THP-1 monocytoid/endothelial cell interaction under shear stress. *Blood* 1999; 94: 2725–2734.

29 Kawasaki T, Dewerchin M, Lijnen HR, Vreys I, Vermylen J, Hoylaerts MF. Mouse carotid artery ligation induces platelet-leukocyte dependent luminal fibrin, required for neointima development. *Circ Res* 2001; 88: 159–166.

30 Østerud B, Rao LV, Olsen JO. Induction of tissue factor expression in whole blood: lack of evidence for the presence of tissue factor expression in granulocytes. *Thromb Haemost* 2000; 83: 861–867.

31 Fay WP, Murphy JG, Owen WG. High concentrations of active plasminogen activator inhibitor-1 in porcine coronary artery thrombi. *Arterioscler Thromb Vasc Biol* 1996; 16: 1277–1284.

32 Friedman RJ, Stemerman MB, Wenz B, Moore S, Gauldie J, Gent M, Tiell ML, Spaet H. The effect of thrombocytopenia on experimental arteriosclerotic lesion formation in rabbits. Smooth muscle cell proliferation and re-endothelialization. *J Clin Invest* 1977; 60: 1191–1201.

33 Berger G, Masse JM, Cramer EM. Alpha-granule membrane mirrors the platelet plasma membrane and contains the glycoproteins Ib, IX, and V. *Blood* 1996; 87: 1385–1395.

34 Harrison P, Cramer EM. Platelet alpha-granules. *Blood Rev* 1993; 7: 52–62. Review.

35 Angelillo A, de Frutos PG, Aparicio C et al. Deficiency or inhibition of Gas6 causes platelet dysfunction and protects against thrombosis. *Nature Medicine* 2001; 7: 215–221.

36 Heijnen HF, Schiel AE, Fijnheer R, Geuze HJ, Sixma JJ. Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood* 1999; 94: 3791–3799.

37 Abrams CS, Ellison N, Budzinski AZ, Shattil SJ. Direct detection of activated platelets and platelet-derived microparticles in humans. *Blood* 1990; 75: 128–138.

38 Jy W, Horstman LL, Arce M, Ahn YS. Clinical significance of platelet microparticles in autoimmune thrombocytopenias. *J Lab Clin Med* 1992; 119: 334–345.

39 George JN, Pickett EB, Saucerman S, McEver RP, Kunicki TJ, Kieffer N, Newman PJ. Platelet surface glycoproteins. Studies on resting and activated platelets and platelet membrane microparticles in normal subjects, and observations in patients during adult respiratory distress syndrome and cardiac surgery. *J Clin Invest* 1986; 78: 340–348.

40 Dachary-Prigent J, Freyssinet JM, Pasquet JM, Carron JC, Nurden AT. Annexin V as a probe of aminophospholipid exposure and platelet membrane vesiculation: a flow cytometry study showing a role for free sulfhydryl groups. *Blood* 1993; 81: 2554–2565.

41 Harding C, Heuser J, Stahl P. Endocytosis and intracellular processing of transferrin and colloidal gold-transferrin in rat reticulocytes: demonstration of a pathway for receptor shedding. *Eur J Cell Biol* 1984; 35: 256–263.

42 Heijnen HF, Debili N, Vainchencker W, Breton-Gorius J, Geuze HJ, Sixma JJ Multivesicular bodies are an intermediate

Cambridge Books Online © Cambridge University Press, 2009

stage in the formation of platelet alpha-granules. *Blood* 1998; 91: 2313–2325.
43 Majerus PW. Inositol phosphate biochemistry. *Annu Rev Biochem* 1992; 61: 225–50.
44 Wang F, Naik UP, Ehrlich YH, Osada S, Ohno S, Kornecki E. Stimulatory antibody-induced activation and selective translocation of protein kinase C isoenzymes in human platelets. *Biochem J* 1995; 311: 401–406.
45 Baldassare JJ, Henderson PA, Burns D, Loomis C, Fisher GJ. Translocation of protein kinase C isozymes in thrombin-stimulated human platelets. Correlation with 1,2-diacylglycerol levels. *J Biol Chem* 1992; 267: 15585–15590.
46 Dalla Via L, Stimamiglio M, Scapin M, Cesaro L, Deana R. Correlation between cytosolic Ca^{2+} concentration, protein phosphorylation and platelet secretion. *Cell Calcium* 1996; 20: 431–440.
47 Vitale ML, Rodriguez Del Castillo A, Trifaro JM. Protein kinase C activation by phorbol esters induces chromaffin cell cortical filamentous actin disassembly and increases the initial rate of exocytosis in response to nicotinic receptor stimulation. *Neuroscience* 1992; 51: 463–474.
48 Vitale ML, Seward EP, Trifaro JM. Chromaffin cell cortical actin network dynamics control the size of the release-ready vesicle pool and the initial rate of exocytosis. *Neuron* 1995; 14: 353–363.
49 Elzagallaai A, Rose SD, Trifaro JM. Platelet secretion induced by phorbol esters stimulation is mediated through phosphorylation of MARCKS: a MARCKS-derived peptide blocks MARCKS phosphorylation and serotonin release without affecting pleckstrin phosphorylation. *Blood* 2000; 95: 894–902.
50 Aderem A. Signal transduction and the actin cytoskeleton: the roles of MARCKS and profilin. *Trends Biochem Sci* 1992; 17: 438–443.
51 Hartwig JH, Thelen M, Rosen A, Janmey PA, Nairn AC, Aderem A. MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. *Nature* 1992; 356: 618–622.
52 Hashimoto Y, Togo M, Tsukamoto K, Horie Y, Watanabe T, Kurokawa K. Protein kinase C-dependent and -independent mechanisms of dense granule exocytosis by human platelets. *Biochim Biophys Acta* 1994; 1222: 56–62.
53 Sloan DC, Haslam RJ. Protein kinase C-dependent and Ca^{2+}-dependent mechanisms of secretion from streptolysin O-permeabilized platelets: effects of leakage of cytosolic proteins. *Biochem J* 1997; 328: 13–21.
54 Jennings LK, Fox JE, Edwards HH, Phillips DR. Changes in the cytoskeletal structure of human platelets following thrombin activation. *J Biol Chem* 1981; 256: 6927–6932.
55 Fox JE. Regulation of platelet function by the cytoskeleton. *Adv Exp Med Biol* 1993; 344: 175–185.
56 Fox JE. The platelet cytoskeleton. *Thromb Haemost* 1993b; 70: 884–893. Review.
57 Hartwig JH, Kwiatkowski DJ. Actin-binding proteins. *Curr Opin Cell Biol* 1991; 3: 87–97.
58 Fox JE, Boyles JK, Reynolds CC, Phillips DR. Actin filament content and organization in unstimulated platelets. *J Cell Biol* 1984; 98: 1985–1991.
59 White JG, Krivit W. An ultrastructural basis for the shape changes induced in platelets by chilling. *Blood* 1967; 30: 625–635.
60 Menche D, Israel A, Karpatkin S. Platelets and microtubules. Effect of colchicine and D2O on platelet aggregation and release induced by calcium ionophore A23187. *J Clin Invest* 1980; 66: 284–291.
61 Berry S, Dawicki DD, Agarwal KC, Steiner M. The role of microtubules in platelet secretory release. *Biochim Biophys Acta* 1989; 1012: 46–56.
62 Yano Y, Sakon M, Kambayashi J et al. Cytoskeletal reorganization of human platelets induced by the protein phosphatase 1/2 A inhibitors okadaic acid and calyculin A. *Biochem J* 1995; 307: 439–449.
63 Bearer EL. Cytoskeletal domains in the activated platelet. *Cell Motil Cytoskeleton* 1995; 30: 50–66.
64 Takenawa T, Itoh T, Fukami K. Regulation of phosphatidylinositol 4,5-bisphosphate levels and its roles in cytoskeletal re-organization and malignant transformation. *Chem Phys Lipids* 1999; 98: 13–22.
65 Marcu MG, Zhang L, Nau-Staudt K, Trifaro JM. Recombinant scinderin, an F-actin severing protein, increases calcium-induced release of serotonin from permeabilized platelets, an effect blocked by two scinderin-derived actin-binding peptides and phosphatidylinositol 4,5-bisphosphate. *Blood* 1996; 87: 20–24.
66 Klenchin VA, Martin TF. Priming in exocytosis: attaining fusion-competence after vesicle docking. *Biochimie* 2000; 82: 399–407.
67 Gerst JE. SNAREs and SNARE regulators in membrane fusion and endocytosis. *Cell Mol Life Sci* 1999; 55: 707–734.
68 Söllner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE. SNAP receptors implicated in vesicle targeting and fusion. *Nature* 1993; 362: 318–324.
69 Rothman JE, Warren G. Implications of the SNARE hypothesis for intracellular membrane topology and dynamics. *Curr Biol* 1994; 4: 220–233.
70 Ludger J and Galli T. Exocytosis: SNAREs drum up! Eur *J Neurosci* 1998; 10: 415–422.
71 Hanson PI, Heuser JE, Jahn R. Neurotransmitter release – four years of SNARE complexes. *Curr Opin Neurobiol* 1997; 7: 310–315.
72 Söllner T, Bennett MK, Whiteheart SW, Scheller RH, Rothman JE. A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* 1993; 75: 409–418.
73 Hanson PI, Roth R, Morisaki H, Jahn R, Heuser JE. Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. *Cell* 1997; 90: 523–535.

Cambridge Books Online © Cambridge University Press, 2009

74 Lin RC, Scheller RH. Structural organization of the synaptic exocytosis core complex. *Neuron* 1997; 19: 1087–1094.
75 Jahn R, Hanson PI. Membrane fusion. SNAREs line up in new environment. *Nature* 1998; 393: 14–15.
76 Fasshauer D, Otto H, Eliason WK, Jahn R, Brunger AT. Structural changes are associated with soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor complex formation. *J Biol Chem* 1997; 272: 28036–28041.
77 Fasshauer D, Bruns D, Shen B, Jahn R, Brunger AT. A structural change occurs upon binding of syntaxin to SNAP-25. *J Biol Chem* 1997; 272: 4582–4590.
78 Rice LM, Brennwald P, Brunger AT. Formation of a yeast SNARE complex is accompanied by significant structural changes. *FEBS Lett* 1997; 415: 49–55.
79 Byrne JH, Kandel ER. Presynaptic facilitation revisited: state and time dependence. *J Neurosci* 1996; 16: 425–435.
80 Ktistakis NT. Signalling molecules and the regulation of intracellular transport. *Bioessays* 1998; 20: 495–504. Review.
81 Fujita Y, Sasaki T, Fukui K et al. Phosphorylation of Munc-18/n-Sec1/rbSec1 by protein kinase C: its implication in regulating the interaction of Munc-18/n-Sec1/rbSec1 with syntaxin. *J Biol Chem* 1996; 27: 7265–7268.
82 Lemons PP, Chen D, Bernstein AM, Bennett MK, Whiteheart SW. Regulated secretion in platelets: identification of elements of the platelet exocytosis machinery. *Blood* 1997; 90: 1490–1500.
83 Polgar J, Reed GL. A critical role for N-ethylmaleimide-sensitive fusion protein (NSF) in platelet granule secretion. *Blood* 1999; 94: 1313–1318.
84 Chen D, Bernstein AM, Lemons PP, Whiteheart S. Molecular mechanisms of platelet exocytosis: role of SNAP-23 and syntaxin 2 in dense core granule release. *Blood* 2000; 95: 921–929.
85 Flaumenhaft R, Furie B, Furie BC. Alpha-granule secretion from alpha-toxin permeabilized, MgATP-exposed platelets is induced independently by H^+ and Ca^{2+}. *J Cell Physiol* 1999; 179: 1–10.
86 Flaumenhaft R, Croce K, Chen E, Furie B, Furie BC. Proteins of the exocytotic core complex mediate platelet alpha-granule secretion. Roles of vesicle-associated membrane protein, SNAP-23, and syntaxin 4. *J Biol Chem* 1999; 274: 2492–2501.
87 Reed GL, Houng AK, Fitzgerald ML. Human platelets contain SNARE proteins and a Sec1p homologue that interacts with syntaxin 4 and is phosphorylated after thrombin activation: implications for platelet secretion. *Blood* 1999; 93: 2617–2626.
88 Bernstein AM, Whiteheart SW. Identification of a cellubrevin/vesicle associated membrane protein 3 homologue in human platelets. *Blood* 1999; 93: 571–579.
89 Chung SH, Polgar J, Reed GL. Protein kinase C phosphorylation of syntaxin 4 in thrombin-activated human platelets. *J Biol Chem* 2000; 275: 25286–25291.
90 Chen D, Lemons PP, Schraw T, Whiteheart SW. Molecular mechanisms of platelet exocytosis: role of SNAP-23 and syntaxin 2 and 4 in lysosome release. *Blood* 2000; 96: 1782–1788.
91 Araki S, Tamori Y, Kawanishi M et al. Inhibition of the binding of SNAP-23 to syntaxin 4 by Munc18c. *Biochem Biophys Res Commun* 1997; 234: 257–262.
92 Lemons PP, Chen D, Whiteheart SW. Molecular mechanisms of platelet exocytosis: requirements for alpha-granule release. *Biochem Biophys Res Commun* 2000; 267: 875–880.
93 Watson EL. GTP-binding proteins and regulated exocytosis. *Crit Rev Oral Biol Med* 1999; 10: 284–306.
94 Zerial M, Stenmark H. Rab GTPases in vesicular transport. *Curr Opin Cell Biol* 1993; 5: 613–620.
95 Schimmoller F, Simon I, Pfeffer SR. Rab GTPases, directors of vesicle docking. *J Biol Chem* 1998; 273: 22161–22164.
96 Novick P, Zerial M. The diversity of Rab proteins in vesicle transport. *Curr Opin Cell Biol* 1997; 9: 496–504.
97 Horiuchi H, Lippe R, McBride HM et al. A novel Rab5 GDP/GTP exchange factor complexed to Rabaptin-5 links nucleotide exchange to effector recruitment and function. *Cell* 1997; 90: 1149–1159.
98 Sasaki T, Kikuchi A, Araki S et al. Purification and characterization from bovine brain cytosol of a protein that inhibits the dissociation of GDP from and the subsequent binding of GTP to smg p25A, a ras p21-like GTP-binding protein. *J Biol Chem* 1990; 265: 2333–2337.
99 Pfeffer SR, Dirac-Svejstrup AB, Soldati T. Rab GDP dissociation inhibitor: putting rab GTPases in the right place. *J Biol Chem* 1995; 270: 17057–17059.
100 Karniguian A, Zahraoui A, Tavitian A. Identification of small GTP-binding rab proteins in human platelets: thrombin-induced phosphorylation of rab3B, rab6, and rab8 proteins. *Proc Natl Acad Sci US A* 1993; 90: 7647–7651.
101 Nagata K, Suzuki T, Okano Y, Hamaguchi M, Nozawa Y. Characterization of a Gly19—>Val mutant of ram p25, a low Mr GTP-binding protein: loss of GTP/GDP-binding activity in the mutated ram p25. *Biochem Biophys Res Commun* 1992; 189: 330–335.
102 Lapetina EG, Lacal JC, Reep BR, Molina y Vedia L. A ras-related protein is phosphorylated and translocated by agonists that increase cAMP levels in human platelets. *Proc Natl Acad Sci USA* 1989; 86: 3131–3134.
103 Cherfils J, Chardin P. GEFs: structural basis for their activation of small GTP-binding proteins. *Trends Biochem Sci* 1999: 306–311. Review.
104 Teramoto H, Salem P, Robbins KC, Bustelo XR, Gutkind JS. Tyrosine phosphorylation of the vav proto-oncogene product links FcepsilonRI to the Rac1–JNK pathway. *J Biol Chem* 1997; 272: 10751–10755.
105 Lorenzo PS, Beheshti M, Pettit GR, Stone JC, Blumberg PM. The guanine nucleotide exchange factor RasGRP is a high-affinity target for diacylglycerol and phorbol esters. *Mol Pharmacol* 2000; 57: 840–846.
106 Baouz S, Jacquet E, Accorsi K et al. Sites of phosphorylation by protein kinase A in CDC25Mm/GRF1, a guanine nucleotide exchange factor for Ras. *J Biol Chem* 2001; 276: 1742–1749.
107 de Rooij J, Zwartkruis FJ, Verheijen MH et al. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* 1998; 396: 474–477.

Cambridge Books Online © Cambridge University Press, 2009

25

Amplification loops: thromboxane generation

Paola Patrignani and Maria G. Sciulli

Department of Medicine and Aging, Division of Pharmacology, 'G. D'Annunzio' University of Chieti, Italy

Inducible and constitutive pathways of prostanoid biosynthesis

Prostanoids [prostaglandin (PG) E_2, $PGF_{2\alpha}$, PGD_2, prostacyclin (PGI_2) and thromboxane (TX) A_2] are a family of biologically active compounds participating in cell–cell communication[1], as a consequence of the activation of membrane associated receptors that belong to the G-protein-coupled rhodopsin-type family[2]. Their biosynthesis is triggered by different types of stimuli that may be classified primarily according to the time employed to induce detectable levels of prostanoids in the surrounding milieu of the cell. Thus, a rapid and delayed prostanoid biosynthesis can occur[1] (Fig. 25.1). The coordinated activity of three consecutive enzymatic steps is involved in both rapid and delayed prostanoid biosynthesis: (i) the release of arachidonic acid (AA) from membrane phospholipids carried out by phospholipase(PL)s, primarily PLA_2s[1,3], (ii) the transformation of AA to the unstable endoperoxide PGH_2 by prostaglandin H synthases[4] and (iii) its metabolization to the different prostanoids by terminal synthases which have different structures and exhibit a cell- and tissue-specific distribution[5–7]. What differentiate these two pathways of prostanoid biosynthesis is the involvement of an enzymatic machinery constitutively expressed (for rapid biosynthesis) or induced in response to inflammatory and mitogenic stimuli (for delayed biosynthesis). Two isoforms of prostaglandin H synthase have been identified, COX-1 and COX-2, that catalyse the same reactions, i.e. the conversion of AA to PGG_2 by their cyclooxygenase activity.

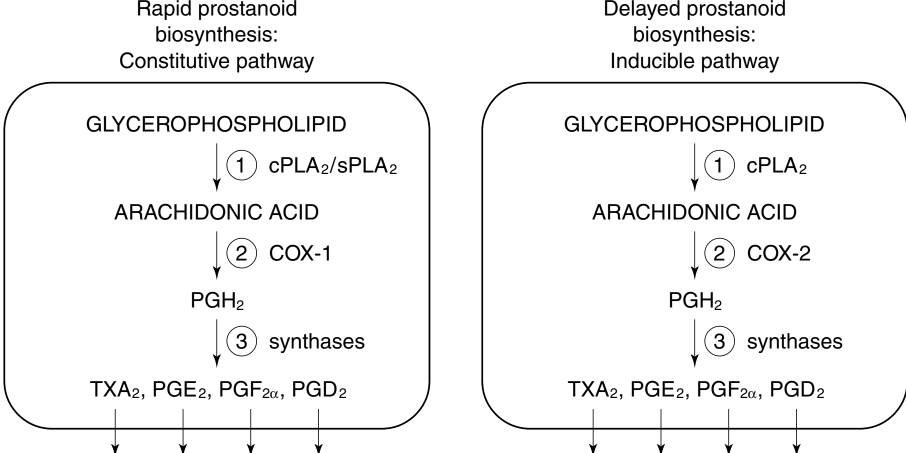


Fig. 25.1. Rapid and delayed pathways of prostanoid biosynthesis. The coordinated activity of three consecutive enzymatic steps are involved in both rapid and delayed prostanoid biosynthesis: (i) the release of arachidonic acid from membrane glycerophospholipids by the secretory and/or cytosolic phospholipaseA_2s ($sPLA_2$ and $cPLA_2$, respectively); (ii) the conversion of arachidonic acid to PGH_2 by the activity of the two isoforms of PGH synthase, COX-1 and COX-2; (iii) the transformation of PGH_2 to different prostanoids (PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2 and TXA_2) by specific synthases.

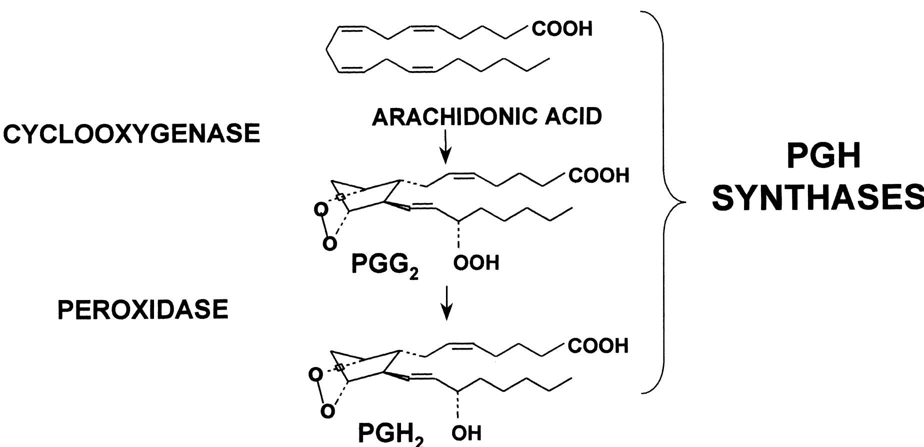


Fig. 25.2. The cyclooxygenase and peroxidase reactions catalyzed by the prostaglandin (PG) H synthases. Arachidonic acid is oxygenated to PGG_2 by the cyclooxygenase activity of PGH synthases (i.e. COX-1 and COX-2); PGG_2 is then reduced to PGH_2 by their peroxidase activity.

Table 25.1. Expression of COX-1 and COX-2

COX-1	COX-2
Chromosome 9	Chromosome 1
Constitutive, found in all tissues; highly expressed in stomach, kidney and platelets	Predominantly inducible in response to inflammatory and mitogenic stimuli in macrophages/ monocytes, synoviocytes, fibroblasts, vascular cells
Can increase from 2- to 4-fold	
	Constitutive in kidney, brain, uterus, vascular cells
	Can increase from 10- to 20-fold

and then the reduction of PGG_2 to PGH_2 by their peroxidase activity[4,8] (Fig. 25.2). Apart from platelets that only contain COX-1[9], most nucleated cells have the gene for COX-2 which can be expressed in response to inflammatory and mitogenic stimuli[4,10] (Table 25.1). COX-1 displays the characteristics of a 'housekeeping gene' and is constitutively expressed in virtually all tissues. It is mainly utilized in the immediate biosynthesis of prostanoids, which occurs within several minutes after stimulation with Ca^{2+} mobilizers. Differently, the inducible COX-2 is an absolute requirement for delayed prostanoid biosynthesis, which lasts for several hours following proinflammatory stimuli.

It has been suggested that the two COX isozymes are differently coupled with specific terminal synthases. For instance, rat peritoneal macrophages produce TXA_2 and PGD_2 through COX-1 in the Ca^{2+} ionophore A23187-induced immediate response and PGE_2 and PGI_2 through COX-2 in the lipopolysaccharide (LPS)-induced delayed response[11,12]. TXA_2 generation by activated platelets depends entirely on COX-1[9,13], whereas PGE_2 production by osteoblasts occurs predominantly through COX-2 irrespective of the co-presence of COX-1[14]. By co-expression of the various prostanoid–biosynthetic enzymes, it has been recently shown that prostanoid synthases are classified into three categories in terms of their localization and COX isozyme preference: (i) the perinuclear enzymes which prefer COX-2 [TXA_2 synthase (TXAS), PGI_2 synthase (PGIS) and membrane-bound PGE_2 synthase (mPGES)], (ii) the cytosolic enzyme which prefers COX-1 [cytosolic PGES (cPGES)] and (iii) the translocating enzyme which utilizes both COX isozymes depending on the stimulus [hematopoietic PGD_2 synthase (H-PGDS)][7]. Alteration in AA supply through endogenous and exogenous routes significantly affects the coupling between COX isozymes and terminal prostanoid synthases. TXAS which resides in the endoplasmic reticulum and perinuclear membranes, preferentially utilizes COX-2 over COX-1 when limited amounts of AA are supplied and can efficiently metabolize COX-1-derived PGH_2 when AA supply is increased[7]. Thus, the coupling of TXAS to COX isozymes is substrate-dependent. In platelets, a coupling between COX-1 and TXAS is promoted for the high availability of free AA upon receptor stimulation.

The inducible pathway of prostanoid biosynthesis causes an intense and localized increase in prostanoid formation that may be rapidly shut up for the instable nature of COX-2 mRNA and protein[15].

A condition where the constitutive pathway of prostanoid biosynthesis plays an important role in the local amplification of cell activation, is exemplified by the bio-

Cambridge Books Online © Cambridge University Press, 2009

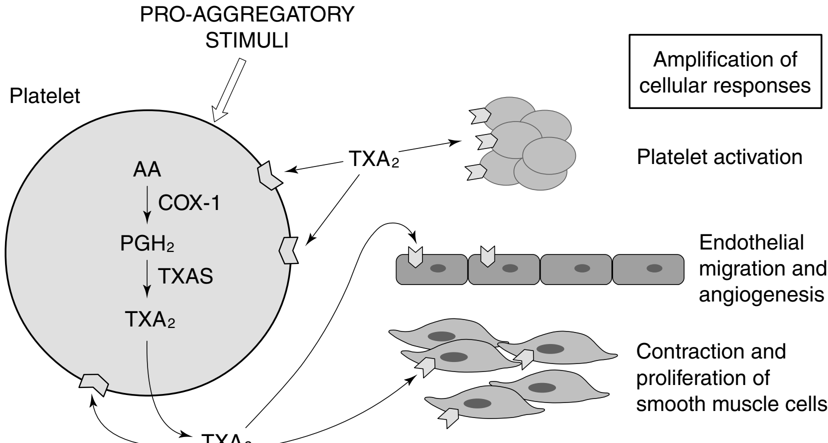


Fig. 25.3. Amplification of cellular responses by platelet-derived TXA_2. Platelet TXA_2, produced in response to pro-aggregatory stimuli, is responsible for the propagation of the initial activation signal to adjacent platelets, vascular smooth muscle cells and endothelial cells, through the interaction with TP receptors.

synthesis of TXA_2 in platelets[16,17] (Fig. 25.3). TXA_2, produced in response to other stronger, primary agonists, such as thrombin and ADP, causes the propagation of the initial activation signal to adjacent platelets, by inducing further platelet activation and TXA_2 formation. TXA_2 is also a potent trigger of vascular smooth muscle cell contraction and proliferation and it has been recently shown to be a mediator of endothelial migration and angiogenesis[18–20] (Fig. 25.3).

TXA_2 biosynthesis in platelets

TXA_2 is the major product of AA in platelets[16,21]. Its biosynthesis is a rather unique example of prostanoid release largely dependent on enhanced substrate availability[22]. Thus, the activation of PLs is a rate-limiting step in TXA_2 production. Once released, AA is offered to COX-1 for its transformation to PGH_2 that is, then, isomerized to TXA_2 by the activity of TXAS[4,8,23,24].

Similarly to all prostanoids, TXA_2 is rapidly metabolized in vivo into biologically inactive compounds. TXA_2 is chemically unstable and is rapidly transformed non-enzymatically to its hydrolysis product, TXB_2[21] (Fig. 25.4); this can then undergo enzymatic metabolism by β-oxidation, resulting in the formation of 2,3-dinor-TXB_2, and by dehydration of the hemiacetal alcohol group at C-11, resulting in the formation of 11-dehydro-TXB_2, the most abundant of a series of metabolites with a Δ-lactone ring structure[25] (Fig. 25.4). These enzymatic metabolites circulate in plasma at low concentrations and are excreted in urine[26–28]. The measurement of these metabolites in

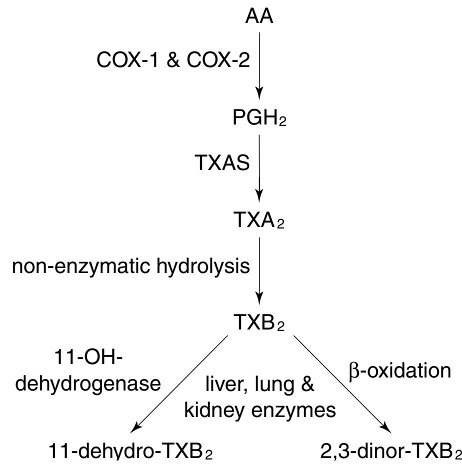


Fig. 25.4. Metabolism of TXA_2. TXA_2, formed by the activity of PGH synthases (COX-1 and COX-2) and TXA_2 synthase (TXAS), is rapidly transformed non-enzymatically to its hydrolysis product TXB_2 which can then undergo two major enzymatic pathways of metabolism: β-oxidation, resulting in the formation of 2,3-dinor-TXB_2, and dehydrogenation of the hemiacetal alcohol group at C-11, resulting in the formation of 11-dehydro-TXB_2.

plasma and urine provides a reliable indication of the systemic biosynthesis of TXA_2. Studies with low-dose aspirin have demonstrated that platelets are the major source of both metabolites in urine[29].

It is interesting to note that the maximal biosynthetic capacity of human platelets to produce TXA_2 when challenged in vitro by endogenous thrombin exceeds, by several thousandfold, the actual rate of TXA_2 biosynthesis

Cambridge Books Online © Cambridge University Press, 2009

in vivo, as reflected by the measurement of urinary excretion of 2,3-dinor-TXB_2 and 11-dehydro-TXB_2[26]. This may explain, at least in part, the unusual requirement of virtually complete suppression of platelet COX-1 activity in order to detect an antithrombotic efficacy[30]. In fact, the relationship between the pharmacological inhibition of platelet COX-1-dependent biosynthetic capacity ex vivo, as assessed by the measurement of TXB_2 production during whole blood clotting, and that of the actual rate of TXA_2 biosynthesis in vivo, as assessed by the measurement of its major enzymatic urinary metabolites, is not linear. Thus, inhibition of TXA_2 biosynthesis in vivo occurs when platelet COX-1 activity, assessed ex vivo, is reduced $>95\%$[31].

Enzymes involved in TXA_2 biosynthesis

Phospholipases (PLs)

The release of AA from platelet membrane glycerophospholipids can occur by two major pathways: PLA_2 and PLC/diacyl glycerol lipase. However, there is substantial evidence to indicate that the activation of PLA_2 is the most important pathway[32,33]. Platelets contain a low (14 kDa) and a high (85 kDa) molecular weight PLA_2, localized in α granules and cytosol, respectively. They are known as secretory PLA_2 ($sPLA_2$)[34] and cytosolic PLA_2 ($cPLA_2$)[35], respectively. PLA_2s hydrolyze the *sn*-2 fatty acyl ester bond of glycerophospholipids, producing non-esterified fatty acids, often represented by AA, and lysophospholipids. The increase in intracellular Ca^{2+} (i.e. μmolar range for $cPLA_2$ and mmolar range for $sPLA_2$) by receptor-operated events is an essential requirement for PLA_2 activation[3]. This event induces the translocation of $cPLA_2$ from the cytosol to cellular membranes[36,37]. Moreover, $cPLA_2$ phosphorylation at Ser^{505}, residing within a mitogen-activated protein kinase (MAPK) consensus sequence (Pro–Leu–Ser^{505}–Pro), causes a severalfold increase of its catalytic activity[37,38]. In thrombin- and collagen-stimulated platelets, p38 kinase, a member of MAPK family, is involved in the early phosphorylation of $cPLA_2$[39]. On the other hand, $cPLA_2$ phosphorylation via a protein kinase C (PKC)/p42/$p44^{MAPK}$-dependent pathway takes place in platelets treated with phorbol esters and Ca^{2+} ionophore[40].

$sPLA_2$ can be released from platelets in response to various stimuli and it has been reported to activate platelets through its binding to a glycophosphatidylinositol (GPI)-anchored platelet-membrane heparan sulfate proteoglycan. This may induce the stimulation of AA metabolism, protein tyrosine phosphorylation and Ca^{2+}-mobilization[41]. However, the involvement of $sPLA_2$ in platelet AA release is not unequivocally accepted[42].

COX-1

Similarly to COX-2, COX-1 is a homodimer of ~70 kDa subunits. Each subunit contains one molecule of heme and comprises three domains: an epidermal growth factor, a membrane-binding and a catalytic domain. The cyclooxygenase and the peroxidase active sites are located in the catalytic domain and are separated by the heme prosthetic group. Tyr-385 residue, at the top of the cyclooxygenase active site, is positioned perfectly to react with AA and to remove the 13-pro-*S*-hydrogen, i.e. the first step in the formation of PGG_2 from AA. Then, PGH_2 is produced via the peroxidase-dependent reduction of PGG_2 peroxyl radical[8] (Fig. 25.2).

Because circulating platelets lack a nucleus and consequently cannot synthesize mRNA, the involvement of constitutively expressed COX-1 in TXA_2 biosynthesis has been assumed. This is supported by cDNA cloning and immunoaffinity purification of only COX-1 from human platelets[43]. In contrast, it has been recently reported that circulating platelets from healthy subjects express COX-2 protein and mRNA, as assessed by Western blot and reverse transcription coupled polymerase chain reaction (RT-PCR), respectively[44]. However, in highly purified human platelet suspensions (containing <0.5 leukocytes per 1000 platelets), analysed immediately after isolation to avoid any ex vivo expression of COX-2 in contaminating monocytes, we failed to detect COX-2 while COX-1 was readily detectable (Fig. 25.5). The involvement of COX-1 in platelet TXB_2 biosynthesis is further supported by the finding that its production is spared by concentrations of NS-398 (a selective COX-2 inhibitor) that completely inhibit monocyte COX-2 activity[9]. The results of over 50 clinical trials showing saturation of the antithrombotic efficacy of aspirin at low-doses (50–100 mg daily)[30] sustain convincingly the COX-1 dependence on platelet TXA_2 biosynthesis.

Aspirin is the only non-steroidal antiinflammatory drug (NSAID) known to react covalently (and time-dependently) with the cyclooxygenase channel of COX-1 and COX-2 causing a selective acetylation of a specific serine residue (Ser^{529} for human COX-1 and Ser^{516} for human COX-2)[45,46]. Aspirin acetylation of platelet COX-1 results in a persistent blockade of AA oxidation to PGH_2 that is cumulative on repeated low-dose administration[13]. An almost complete suppression ($>95\%$) of platelet COX-1 activity is obtained after 5–7 days of treatment with low-dose aspirin. In contrast, in a nucleated cell, the irreversible inactivation of COX-1 and COX-2 by the drug can be rapidly reversed by *de novo* synthesis of the enzymes[47].

Cambridge Books Online © Cambridge University Press, 2009

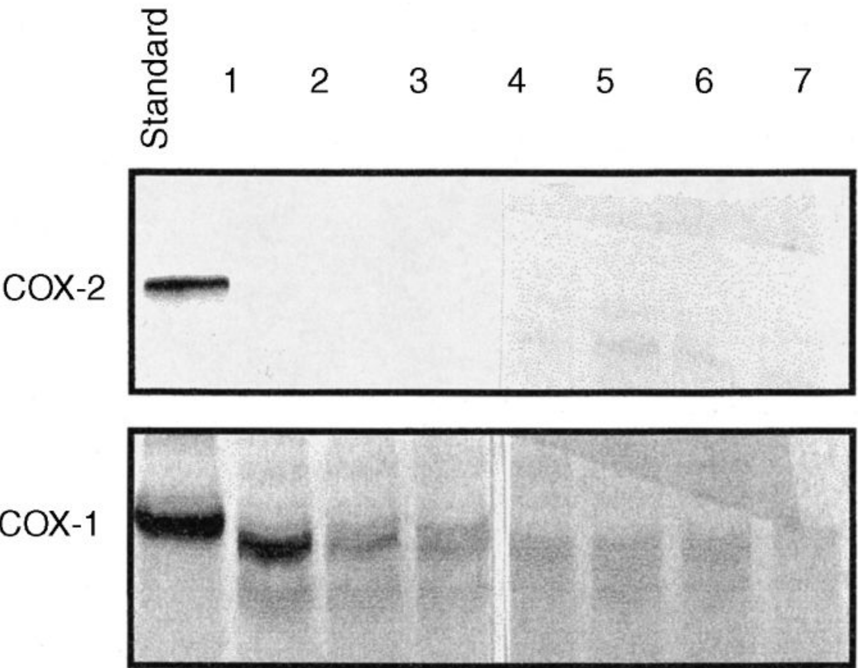


Fig. 25.5. Western blot analysis of COX-1 and COX-2 in human platelets. Platelets were isolated from citrated human whole blood drawn from seven healthy volunteers. COX-1 and COX-2 levels were analysed by SDS-polyacrylamide gel electrophoresis and immunoblotting techniques, using rabbit polyclonal antibodies directed against COX-1 or a carboxyl-terminal sequence present only in human COX-2. In the first lane, COX-1 and COX-2 standard proteins are shown; the numbers (1–7) indicate the 7 healthy volunteers.

TXAS

TXAS catalyses the isomerization of PGH_2 to TXA_2 that is accompanied with the formation of 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and malondialdehyde (MDA) at a ratio of 1:1:1. However, the biological functions of HHT and MDA are unclear. TXAS, a protein with a molecular weight of 58.8 kDa, is a member of the cytochrome P450 superfamily and is associated with endoplasmic reticulum and perinuclear membranes[48]. Human TXAS was assigned as CYP 5A1. TXAS is synthesized by numerous tissues, including kidney and brain. In blood derived cells, TXAS has been detected in macrophages and platelets[49]. Its mRNA and protein are present in normal megakaryocytes[50]. Cloning of TXAS has revealed that the promoter region has a TATA box and several potential binding sites for transcription factors and a glucocorticoid response element[51]. TXAS is expressed during differentiation of megakaryocytes through the regulation of $p45^{NF-E2}$, a transcription factor that plays a crucial role in the regulation of erythroid gene expression. However, megakaryocyte TXAS expression and TXA_2 biosynthesis do not play a role in the platelet production process[50]. This is supported by the finding that patients with TXAS deficiency present bleeding disorders in the absence of thrombocytopenia[52]. A variation in the promoter region of TXAS, i.e. a T to G substitution at nucleotide-386, has been reported[53]. In vitro studies have shown that the G(-386) allele presented enhanced promoter activity compared to the T(-386) allele. A higher G(-386) allele frequency of TXAS gene has been demonstrated in Japanese patients with acute myocardial infarction than those with angina pectoris[53]. Moreover, 11 polymorphisms in human TXAS gene, including 8 missense mutations, have been recently identified[54]. However, the functional consequence of these variations of TXAS gene remains to be studied.

Pharmacological inhibition of TXAS causes the increase in the formation of PGE_2, $PGF_{2\alpha}$ and PGD_2 by activated platelets and PGI_2 by the vascular endothelium and blood leukocytes[55–57]. However, TXAS blockade is also associated with accumulation of PG endoperoxides (PGG_2 and PGH_2) that can occupy and activate platelet TXA_2 receptors (TPs). These complex biochemical changes transduce opposing signals, resulting in mild and inconsistent impairment of platelet aggregation[55]. Combined TXAS inhibitors and TP antagonists have theoretical advantages over existing antiplatelet agents by virtue of increasing vascular PGI_2

Cambridge Books Online © Cambridge University Press, 2009

Table 25.2. Alteration of platelet TXA_2 biosynthesis in humans

Enhanced TXA2 biosynthesis		Reference number
Unstable angina		60–62
Acute cerebral ischemia and acute ischemic stroke		69, 70
Chronic phase after cerebral ischemia and intracerebral hemorrhage		71
Type IIa hypercholesterolemia		63
Diabetes mellitus, type I and II		64, 65
Homozygous homocystinuria		67
Smokers		68
Essential thrombocythemia		72
Polycythemia vera		73
Reduced TXA_2 biosynthesis	**Mechanism**	**Reference number**
Bleeding disorders	↓ AA release	74
	↓ COX-1 protein levels and COX-1 activity	75, 76
Uremia	↓ COX-1 activity	77
Liver cirrhosis	↓ Agonist-induced transmembrane signalling	78

biosynthesis and affecting the interaction of PGH_2 with the platelet TPs. Whether these theoretical advantages will translate into measurable improvements over existing antiplatelet agents remains to be tested in comparative clinical trials.

Alteration of platelet thromboxane biosynthesis in humans

The biosynthesis of platelet TXA_2 can be evaluated by the measurement of TXB_2 production in response to various agonists (e.g. ADP, collagen, arachidonate) added in vitro to washed platelets or platelet rich plasma (PRP). This is a measure of 'capacity', which indicates what platelets can do when challenged by rather intense stimuli in a closed artificial system. Measurement of TXB_2 production during whole blood clotting is also a capacity index related to virtually maximal conversion of platelet AA through the COX-1 pathway in response to endogenously formed thrombin[58]. The relevance of these capacity indexes to the actual occurrence of platelet activation in vivo is still unknown. In contrast, the measurement of urinary excretion of major enzymatic metabolites of TXB_2 is thought to be a reliable index of platelet function in vivo. In fact: (i) 11-dehydro-TXB_2 and 2,3-dinor-TXB_2 are excreted at low rates under physiological circumstances[26] (i.e. low background 'noise' unaffected by sampling-related artefacts), (ii) their excretion increases linearly with increased rates of entry of TXB_2 into the systemic circulation[26,27] and (iii) they are largely derived from platelet TXA_2 biosynthesis in vivo[29]. The contribution of platelets to enhanced urinary TXB_2 metabolite excretion, in different clinical conditions, can be verified by assessing the extent of its inhibition and the time of its recovery after the daily administration of low-dose aspirin for 5–7 consecutive days. An almost complete suppression of platelet COX-1 activity, as assessed by measuring serum TXB_2 production, by low-dose aspirin is associated with a 70–80% reduction of urinary 11-dehydro-TXB_2 and 2,3-dinor-TXB_2 excretion[29]; after discontinuing aspirin administration, urinary TXB_2 metabolite excretion returns to pre-drug levels within 10–14 days[29], which is consistent with platelet turnover.

Episodic increases in TXB_2 metabolite excretion have been detected in patients with unstable angina[59–62]. Platelet activation in this setting is not merely related to the presence of atherosclerotic lesions in the arterial wall, but is an acute process most likely triggered by sudden rupture of the plaque[59]. In fact, the levels of TXB_2 metabolites were unchanged in patients with chronic stable angina[60] and peripheral vascular disease[62]. In contrast, a relatively reproducible and persistent increase in TXB_2 metabolite excretion has been demonstrated in association with cardiovascular risk factors (i.e. hypercholesterolemia, diabetes mellitus, hypertension, homocystinuria or smoking)[63–68]. The results of these studies suggest that the occurrence of arterial disease *per se* is not a trigger of plate-

Cambridge Books Online © Cambridge University Press, 2009

let activation in vivo. Rather, the rate of TXA_2 biosynthesis appears to reflect the influence of coexisting disorders. Thus, it has been proposed that TXA_2-mediated amplification of platelet activation in response to plaque fissuring may represent a link between different risk factors and the occurrence of thrombotic complications[66]. In Table 25.2, different clinical conditions characterized by enhanced TXA_2 biosynthesis in vivo are listed.

Reduced biosynthesis of platelet TXA_2 in vitro has been found in patients with bleeding disorders (Table 25.2). The mechanism of this defect may involve an impaired release of AA from phospholipids[74] or a COX-1 deficiency, i.e. undetectable protein levels or impaired catalytic activity[75–77]. Moreover, platelets from patients with liver cirrhosis produce reduced levels of TXA_2 in response to thrombin and collagen as a consequence of an impairment of transmembrane signalling through a molecular mechanism not yet identified[78]. Mild to moderate bleeding disorders have been associated with defective Ca^{2+} ionophore A23187-induced platelet aggregation without impaired TXA_2 production[79]. A defective or reduced platelet aggregation and secretion in response to ADP, collagen, AA and stable TXA_2 (STA_2) has been also detected. The molecular mechanisms of these defects are independent on PKC activation, Ca^{2+} mobilization and myosin light chain (MLC) phosphorylation.

Endogenous modulation of platelet TXA_2 biosynthesis

Platelet TXA_2 biosynthesis is, presumably, turned off through two main mechanisms: (i) the decrease in TXA_2 synthesis for suicide inactivation of COX-1 and TXAS and (ii) the irreversible sticking of platelets associated with the release of their intracellular granules. COX-1 is a self-inactivating enzyme. Both the peroxidase and cyclooxygenase activities have a limited number of catalytic turnovers[8]. Based on the protective effect of various reducing cosubstrates, it has been proposed that active radical intermediates, formed during the peroxidase or cyclooxygenase catalysis, are involved in the irreversible loss of enzyme activity[80]. Similarly, TXAS undergoes suicide inactivation during catalysis, are involved presumably via two mechanisms: (i) a covalent modification of the enzyme due to a tight association with PGH_2 or with an intermediate product not yet identified and (ii) a modification or dissociation of the heme prosthetic group[81]. Moreover, both COX-1 and TXAS activities may be modulated by the cellular levels of lipid hydroperoxides and/or nitric oxide[8,82].

Counterregulatory mechanisms can be evoked by platelet activation[22]. For instance, platelets can induce the synthesis of PGI_2 in adjacent vascular endothelial cells by transcellular metabolism: (i) platelet-derived PGH_2 can be utilized by endothelial PGIS[83], (ii) platelet microparticles or fragments of plasma membrane may deliver their unmetabolized AA content to endothelial cells and induce COX-2 expression via a PKC/MAPK-dependent pathway[84] (Fig. 25.6). Alternatively, activated platelets can cause overexpression of endothelial COX-2 as a consequence of direct cell-cell interaction or growth factor secretion (Fig. 25.6).

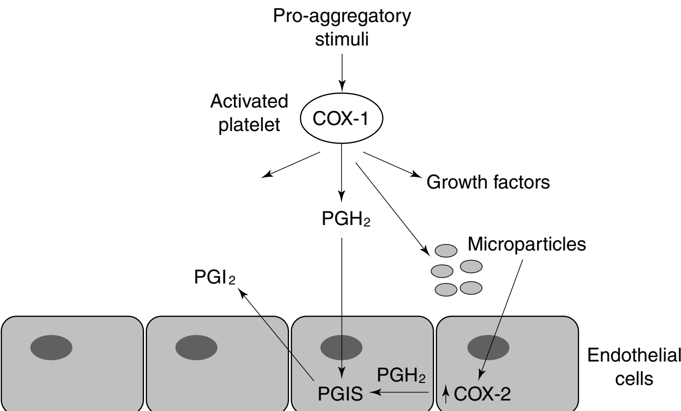


Fig. 25.6. Mechanisms involved in endothelial PGI_2 biosynthesis by activated platelets. PGI_2 synthase (PGIS) can utilize PGH_2, derived from platelet COX-1 and from endothelial COX-2, induced in response to platelet-derived growth factors and microparticles, to produce PGI_2.

PGI_2 has been proposed to be an atheroprotective mediator for its inhibitory effects on proliferation and contraction of vascular smooth muscle cells and platelet activation[85]. The involvement of PGI_2 in the control of platelet function in vivo is suggested by the finding that deletion of the PGI_2 receptor, the IP, results in an increased response to thrombotic stimuli in the mouse[86].

TXA_2 receptors (TPs)

TXA_2 acts on a receptor belonging to the heptahelical superfamily of G protein-coupled receptors[2]. TP purified from human platelets consists of a broad protein band of 57 kDa[87]. Although pharmacological studies, using different ligands, suggest heterogeneity of TPs[88], a single gene has been cloned from a placental library[89]. Deletion of this gene renders mouse platelets unresponsive to thromboxane analogues and abolishes the pressor response to infusion of TP agonists[90].

Two splice variants of TP have been identified, α and

Cambridge Books Online © Cambridge University Press, 2009

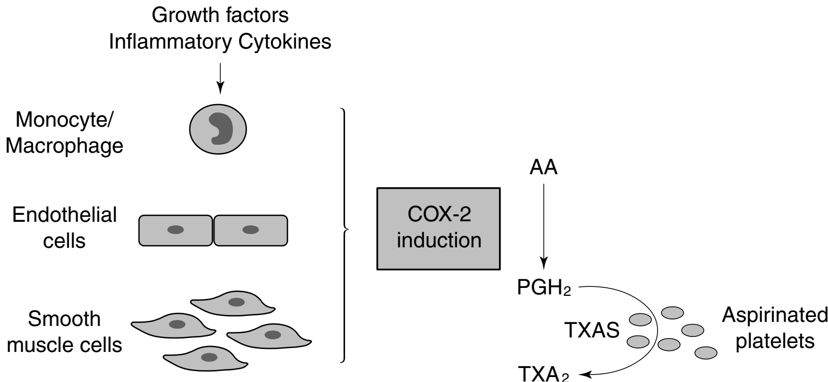


Fig. 25.7. Aspirin-treated platelets metabolize PGH_2 derived from vascular cells and macrophages into TXA_2. COX-2 induction in activated endothelial cells, smooth muscle cells and macrophages may contribute to TXA_2 biosynthesis by providing PGH_2 to the TXAS of aspirinated platelets.

β[89,91]. They differ only in the carboxyl-terminal tail. TPβ isoform has a longer carboxyl-terminal extension than TPα isoform. Although mRNA for both isoforms exists in platelets, only TPα protein has been detected[92]. In contrast, both isoforms are expressed in endothelial and vascular smooth muscle cells[93]. The two isoforms of the TP differ in their rates of agonist-induced desensitization and propensity to couple with G proteins[94,95]. Thus, TPβ, but not TPα, undergoes agonist-induced internalization in several different cell types, presumably, through phosphorylation in a region containing three serine residues of its C-tail. Activation of TP isoforms causes the increase of phosphoinositide-specific phospholipase C (PLC) activity via the coupling with pertussis-insensitive GTP binding protein, G_q[96]. In contrast, only TPα signals via G_h to induce PLC-dependent IP_3 formation[95].

Pharmacological studies using the ligand I-BOP have evidenced the presence of two binding sites (high and low affinity) on platelet membranes. The interaction with the low affinity binding site induces secretion and aggregation, whereas that with the high affinity binding site induces shape change[97]. Since only TPα has been detected in human platelets, these two binding sites seem to represent different states of the same TP isoform.

Impaired coupling of TP receptor to G protein, as a consequence of a single aminoacid substitution (Arg[60] to Leu) in the first cytoplasmatic loop of the receptor, has been associated with a dominantly inherited bleeding disorder[98]. In fact, ligand activation of mutant TP receptor, expressed in Chinese hamster ovary (CHO) cells, has been shown to cause a decreased formation of second messengers despite a normal binding affinity[98].

A newly synthesized TP antagonist, S18886, which is devoid of partial agonist activity, has been shown to retard atherogenesis in the apoE-knockout mouse[99]. Since aspirin is ineffective, the possible involvement of platelet TP blockade is, presumably, excluded. Thus, the antagonist may have interfered with the activation of TPs expressed in other cells relevant to atherosclerosis (i.e. vascular smooth muscle cells, macrophages). Moreover, the possible prevention of isoprostane-dependent TP activation by S18886 cannot be excluded. Isoprostanes are PG isomers formed by free-radical catalysed arachidonate peroxidation[100]. 8-iso-$PGF_{2\alpha}$, one of the most abundant F_2-isoprostanes formed in vivo in humans, has been shown to induce platelet and vascular smooth muscle cell activation through the interaction with TPs[101].

Aspirin-insensitive TXA_2 biosynthesis

Studies performed in vitro have demonstrated that aspirin-treated platelets restore their capacity to synthesize TXA_2 if they are incubated with thrombin-stimulated endothelial cells[47]. Since the extent of TXA_2 production was proportional to the amount of endothelial COX-2 expression, it has been suggested that COX-2-dependent PGH_2 can be utilized by the TXAS of aspirinated platelets to produce TXA_2 (Fig. 25.7). Differently from platelets, which are persistently inactivated by aspirin, endothelial cells can rapidly (2–4 h) recover the aspirin-dependent irreversible inhibition of COX-2 activity after treatment with phorbol esters or interleukin (IL)-1α, due to *de novo* synthesis of COX-2[47]. These in vitro findings suggest that after a single daily administration of the short-lived aspirin, extraplatelet TXA_2 biosynthesis can be restored through the induction of COX-2 in nucleated cells (i.e. vascular cells, monocytes/macrophages) in response to a local inflam-

Cambridge Books Online © Cambridge University Press, 2009

matory milieu (Fig. 25.7). This phenomenon may be operative in vivo. In fact, in a subset of patients with unstable angina treated with low-dose aspirin to almost completely block platelet COX-1 activity, enhanced TXA_2 biosynthesis in vivo, as reflected by the urinary excretion of 11-dehydro-TXB_2 and 2,3-dinor-TXB_2, has been demonstrated[62]. The involvement of extraplatelet sources, possibly expressing COX-2, has been suggested by the finding that indobufen, an antiplatelet drug that largely inhibits both platelet COX-1 and monocyte COX-2 at therapeutic plasma concentrations, causes a more profound reduction of TXA_2 biosynthesis than low-dose aspirin, a selective inhibitor of platelet COX-1, in this setting[102]. Thus, COX-2 induction in plaque monocyte/macrophages or activated vascular cells may contribute to aspirin-insensitive TXA_2 biosynthesis in patients with acute coronary syndromes by generating PGH_2 as a substrate for the TXAS of the same cell or by providing PGH_2 to the TXAS of aspirinated platelets (Fig. 25.7).

Conclusions

COX-1-dependent TXA_2 biosynthesis produced by platelets in response to other primary agonists plays an important role in the local amplification of cell activation. This is demonstrated by the results of randomized clinical trials with aspirin showing that the antithrombotic effect is saturable at doses (50–100 mg/day) causing a selective inhibition of platelet COX-1 activity[30].

Aspirin-insensitive thromboxane biosynthesis occurring episodically in patients with acute coronary syndromes may provide a mechanism for the formation of a potent agonist of platelet and vascular TP receptors, possibly contributing to a number of clinical aspirin failures. The availability of potent and long-lasting TP antagonists[103] and selective COX-2 inhibitors (coxibs)[104] offers the opportunity to test this hypothesis.

References

1 Fitzpatrick FA, Soberman R. Regulated formation of eicosanoids. *J Clin Invest* 2001; 107: 1347–1351.
2 Narumiya S, FitzGerald GA. Genetic and pharmacological analysis of prostanoid receptor function. *J Clin Invest* 2001; 108: 25–30.
3 Dennis EA. Phospholipase A_2 in eicosanoid generation. *Am J Respir Crit Care Med* 2000; 161: S32–S35.
4 Smith WL, Langenbach R. Why there are two cyclooxygenase isozymes. *J Clin Invest* 2001; 12: 1491–1495.
5 Jakobsson PJ, Thoren S, Morgenstern R, Samuelsson B. Identification of the human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. *Proc Natl Acad Sci USA* 1999; 96: 7220–7225.
6 Tanioka T, Nakatani Y, Semmyo N, Murakami M, Kudo I. Molecular identification of cytosolic prostaglandin E_2 synthase that is functionally coupled with cyclooxygenase-1 in immediate prostaglandin E_2 biosynthesis. *J Biol Chem* 2000; 275: 32775–32782.
7 Ueno N, Murakami M, Tanioka T, Fujimori K, Urade Y, Kudo I. Coupling between cyclooxygenases, terminal prostanoid synthases and phospholipase A_2s. *J Biol Chem* 2001 (in press).
8 Marnett LJ, Rowlinson SW, Goodwin DC, Kalgutkar AS, Lanzo CA. Arachidonic acid oxygenation by COX-1 and COX-2. Mechanisms of catalysis and inhibition. *J Biol Chem* 1999; 274: 22903–22906.
9 Patrignani P, Sciulli MG, Manarini S, Santini G, Cerletti C, Evangelista V. COX-2 is not involved in thromboxane biosynthesis by activated human platelets. *J Physiol Pharmacol* 1999; 50: 661–667.
10 Crofford LJ. COX-1 and COX-2 tissue expression: implications and predictions. *J Rheumatol* 1997; 24 (Suppl. 49): 15–19.
11 Naraba H, Murakami M, Matsumoto H et al. Segregated coupling of phospholipases A_2, cyclooxygenases, and terminal prostanoid synthases in different phases of prostanoid biosynthesis in rat peritoneal macrophages. *J Immunol* 1998; 160: 2974–2982.
12 Brock TG, McNish RW, Peters-Golden M. Arachidonic acid is preferentially metabolized by cyclooxygenase-2 to prostacyclin and prostaglandin E_2. *J Biol Chem* 1999; 274: 11660–11666.
13 Patrignani P, Filabozzi P, Patrono C. Selective cumulative inhibition of platelet thromboxane production by low-dose aspirin in healthy subjects. *J Clin Invest* 1982; 69: 1366–1372.
14 Chen QR, Miyaura C, Higashi S et al. Activation of cytosolic phospholipase A_2 by platelet-derived growth factor is essential for cyclooxygenase-2-dependent prostaglandin E_2 synthesis in mouse osteoblasts cultured with interleukin-1. *J Biol Chem* 1997; 272: 5952–5958.
15 Ristimaki A, Garfinken S, Wessendorf J, Maciag T, Hla T. Induction of cyclooxygenase-2 by interleukin-1 alpha. Evidence for post-transcriptional regulation. *J Biol Chem* 1994; 269: 11769–11775.
16 Majerus PW. Arachidonic metabolism in vascular disorders. *J Clin Invest* 1983; 72: 1521–1525.
17 FitzGerald GA. Mechanisms of platelet activation: TXA_2 as an amplifying signal for other agonists. *Am J Cardiol* 1991; 68: 11B-15B.
18 Ellis EF, Oelz O, Roberts LJ, Oates JA. Coronary arterial smooth muscle contraction by a substance released from platelets: evidence that it is thromboxane A_2. *Science* 1976; 193: 1135–1137.
19 Dorn GW 2nd. Role of thromboxane A_2 in mitogenesis of vascular smooth muscle cells. *Agents Actions* 1997; 48 (Suppl): 42–62.

Cambridge Books Online © Cambridge University Press, 2009

20 Daniel TO, Liu H, Morrow JD, Crews BC, Marnett LJ. Thromboxane A_2 is a mediator of cyclooxygenase-2-dependent endothelial migration and angiogenesis. *Cancer Res* 1999; 59: 4574–4577.

21 Hamberg M, Svensson J, Samuelsson B. Thromboxanes. A new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc Natl Acad USA Sci* 1975; 72: 2994–2998.

22 Patrono C, Patrignani P, Rodriguez LA. Cyclooxygenase-selective inhibition of prostanoid formation transducing biochemical selectivity into clinical read-outs. *J Clin Invest* 2001; 108: 7–13.

23 Needleman P, Moncada S, Bunting S, Vane JR, Hamberg M, Samuelsson B. Identification of an enzyme in platelet microsomes which generates thromboxane A_2 from prostaglandin endoperoxides. *Nature* 1976; 261: 558–560.

24 Ullrich V, Nüsing R. Thromboxane synthase from isolation to function. *Stroke* 1990; 21 (Suppl. IV): 134–138.

25 Roberts II RJ, Sweetman BJ, Oates JA. Metabolism of thromboxane B_2 in man: identification of twenty urinary metabolites. *J Biol Chem* 1981; 256: 8384–8393.

26 Patrono C, Ciabattoni G, Pugliese F, Pierucci A, Blair I, FitzGerald GA. Estimated rate of thromboxane secretion into the circulation of normal humans. *J Clin Invest* 1986; 72: 590–594.

27 Ciabattoni G, Pugliese F, Davì G, Pierucci A, Simonetti BM, Patrono C. Fractional conversion of thromboxane B_2 to urinary 11-dehydro-TXB_2 in man. *Biochim Biophys Acta* 1989; 992: 66–70.

28 Patrono C, Davì G, Ciabattoni G. Thromboxane biosynthesis and metabolism in relation to cardiovascular risk factors. *Trends Cardiovasc Med* 1992; 2: 15–20.

29 Catella F, FitzGerald GA. Paired analysis of urinary thromboxane B_2 metabolites in humans. *Thromb Res* 1987; 47: 647–656.

30 Patrono C, Coller B, Dalen JE et al. Platelet-active drugs. The relationships among dose, effectiveness, and side effects. *Chest* 1998; 114: 470S-488S.

31 Reilly IA, FitzGerald GA. Inhibition of thromboxane formation in vivo and ex vivo: implications for therapy with platelet inhibitory drugs. *Blood* 1987; 69: 180–186.

32 Mahadevappa VG, Holub BJ. Diacylglycerol lipase pathway is a minor source of released arachidonic acid in thrombin-stimulated human platelets. *Biochem Biophys Res Commun* 1986; 134: 1327–1333.

33 Clark JD, Schievella AR, Nalefski EA, Lin LL. Cytosolic phospholipase A_2. *J Lipid Mediat Cell Signal* 1995; 12: 83–117.

34 Kramer RM, Hession C, Johansen B et al. Structure and properties of a human non-pancreatic phospholipase A_2. *J Biol Chem* 1989; 264: 5768–5775.

35 Kramer RM, Roberts EF, Manetta JV, Hyslop PA, Jakubowski JA. Thrombin-induced phosphorylation and activation of Ca^{2+} sensitive cytosolic phospholipase A_2 in human platelets. *J Biol Chem* 1993; 268: 26796–26804.

36 Clark JD, Lin LL, Kriz RW et al. A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+} dependent translocation domain with homology to PKC and GAP. *Cell* 1991; 65: 1043–1051.

37 McNicol A, Shibou TS. Translocation and phosphorylation of cytosolic phospholipase A_2 in activated platelets. *Thromb Res* 1998; 92: 19–26.

38 Lin LL, Wartmann M, Lin AY, Knopf JL, Seth A, Davìs RJ. $cPLA_2$ is phosphorylated and activated by MAP kinase. *Cell* 1993; 72: 269–278.

39 Kramer RM, Roberts EF, Um SL et al. p38 mitogen-activated protein kinase phosphorylates cytosolic phospholipase A_2 ($cPLA_2$) in thrombin-stimulated platelets. *J Biol Chem* 1996; 271: 27723–27729.

40 Börsch-Haubold AG, Kramer RM, Watson SP. Inhibition of mitogen-activated protein kinase does not impair primary activation of human platelets. *Biochem J* 1996; 318: 207–212.

41 Polgár J, Kramer RM, Um SL, Jakubowski JA, Clemetson KJ. Human group II 14 kDa phospholipase A_2 activates human platelets. *Biochem J* 1997; 327: 259–265.

42 Mounier C, Faili A, Vargaftig BB, Bon C, Hatmi M. Secretory phospholipase A_2 is not required for arachidonic acid liberation during platelet activation. *Eur J Biochem* 1993; 216: 169–175.

43 Takahashi Y, Ueda N, Yoshimoto T et al. Immunoaffinity purification and cDNA cloning of human platelet prostaglandin endoperoxide synthase (cyclooxygenase). *Biochem Biophys Res Commun* 1992; 182: 433–438.

44 Weber A-A, Zimmermann KC, Meyer-Kircharath J, Schror K. Cyclooxygenase-2 in human platelets as a possible factor in aspirin resistance. *Lancet* 1999; 353: 900.

45 Lecomte M, Laneuville O, Ji C, DeWitt DL, Smith WL. Acetylation of human prostaglandin endoperoxide synthase-2 (cyclooxygenase-2) by aspirin. *J Biol Chem* 1994; 269: 13207–13215.

46 Loll PJ, Picot D, Garavito RM. The structural basis of aspirin activity inferred from the crystal structure of inactivated prostaglandin H_2 synthase. *Nat Struct Biol* 1995; 2: 637–643.

47 Karim S, Habib A, Levy-Toledano S, Maclouf J. Cyclooxygenase-1 and -2 of endothelial cells utilize exogenous or endogenous arachidonic acid for transcellular production of thromboxane. *J Biol Chem* 1996; 271: 12042–12048.

48 Haurand M, Ullrich V. Isolation and characterization of thromboxane synthase from human platelets as cytochrome P-450 enzyme. *J Biol Chem* 1985; 260: 15059–15067.

49 Nusing R, Ullrich V. Immunoquantitation of thromboxane synthase in human tissues. *Eicosanoids* 1990; 3: 175–180.

50 Vitrat N, Letestu R, Massè A, Lazar V, Vainchenker W, Debili N. Thromboxane synthase has the same pattern of expression as platelet specific glycoproteins during human megakaryocyte differentiation. *Thromb Haemost* 2000; 83: 759–768.

51 Lee K-D, Baek SJ, Shen R-F. Cloning and characterization of the human thromboxane synthase gene promoter. *Biochem Biophys Res Commun* 1994; 201: 379–387.

52 Mestel F, Oetliker O, Beck E, Felix R, Imbach P, Wagner HP. Severe bleeding associated with defective thromboxane synthetase. *Lancet* 1980; 1: 157.

Cambridge Books Online © Cambridge University Press, 2009

53 Tsujita Y, Kinoshita M, Tanabe T, Iwai N. Role of a genetic variation in the promoter of human thromboxane synthase gene in myocardial infarction. *Atherosclerosis* 2000; 153: 261–262.
54 Chevalier D, Lo-Guidice JM, Sergent E et al. Identification of genetic variants in the human thromboxane synthase gene (CYP5A1). *Mutat Res* 2001; 432: 61–67.
55 FitzGerald GA, Reilly IA, Pedersen AK. The biochemical pharmacology of thromboxane synthase inhibition in man. *Circulation* 1985; 72: 1194–1201.
56 Patrignani P, Filabozzi P, Catella F, Pugliese F, Patrono C. Differential effects of dazoxiben, a selective thromboxane-synthase inhibitor, on platelet and renal prostaglandin-endoperoxide metabolism. *J Pharmacol Exp Ther* 1984; 228: 472–477.
57 FitzGerald GA, Brash AR, Oates JA, Pedersen AK. Endogenous prostacyclin biosynthesis and platelet function during selective inhibition of thromboxane synthase in man. *J Clin Invest* 1983; 72: 1336–1343.
58 Patrono C, Ciabattoni G, Pinca E et al. Low dose aspirin and inhibition of thromboxane B_2 production in healthy subjects. *Thromb Res* 1980; 17: 317–327.
59 Patrono C, Renda G. Platelet activation and inhibition in unstable coronary syndromes. *Am J Cardiol* 1997; 80: 17E-20E.
60 FitzGerald DJ, Roy L, Catella F, FitzGerald GA. Platelet activation in unstable coronary disease. *N Engl J Med* 1986; 315: 983–989.
61 Hamm CW, Lorenz RL, Bleifeld W, Kupper W, Wober W, Weber PC. Biochemical evidence of platelet activation in patients with persistent unstable angina. *J Am Coll Cardiol* 1987; 10: 988–1006.
62 Vejar M, Fragasso G, Hackett D, Lipkin DP, Maseri A, Born GV, Ciabattoni G, Patrono C. Dissociation of platelet activation and spontaneous myocardial ischemia in unstable angina. *Thromb Haemost* 1990; 63: 163–168.
63 Davì G, Averna M, Catalano I et al. Increased thromboxane biosynthesis in type IIa hypercholesterolemia. *Circulation* 1992; 85: 1792–1798.
64 Davì G, Catalano I, Averna M et al. Thromboxane biosynthesis and platelet function in type II diabetes mellitus. *N Engl J Med* 1990; 322: 1769–1774.
65 Alessandrini P, McRae J, Feman S, FitzGerald GA. Thromboxane biosynthesis and platelet function in type I diabetes mellitus. *N Engl J Med* 1988; 319: 208–212.
66 Davì G, Gresele P, Violi F et al. Diabetes mellitus, hypercholesterolemia, and hypertension but not vascular disease per se are associated with persistent platelet activation in vivo. Evidence derived from the study of peripheral arterial disease. *Circulation* 1997; 96: 69–75.
67 Di Minno G, Davì G, Margaglione M et al. Abnormally high thromboxane biosynthesis in homozygous homocystinuria. Evidence for platelet involvement and probucol-sensitive mechanism. *J Clin Invest* 1993; 92: 1400–1406.
68 Nowak J, Murray JJ, Oates JA, FitzGerald GA. Biochemical evidence of a chronic abnormality in platelet and vascular function in healthy individuals who smoke cigarettes. *Circulation* 1987; 76: 6–14.
69 Koudstaal PJ, Ciabattoni G, van Gijn J et al. Increased thromboxane biosynthesis in patients with acute cerebral ischemia. *Stroke* 1993; 24: 219–223.
70 van Kooten F, Ciabattoni G, Patrono C, Schmitz PI, van Gijn J, Koudstaal PJ. Evidence for episodic platelet activation in acute ischemic stroke. *Stroke* 1994; 25: 278–281.
71 van Kooten F, Ciabattoni G, Koudstaal PJ, Dippel DW, Patrono C. Increased platelet activation in the chronic phase after cerebral ischemia and intracerebral hemorrhage. *Stroke* 1999; 30: 546–549.
72 Rocca B, Ciabattoni G, Tartaglione R et al. Increased thromboxane biosynthesis in essential thrombocythemia. *Thromb Haemost* 1995; 74: 1225–1230.
73 Landolfi R, Ciabattoni G, Patrignani P et al. Increased thromboxane biosynthesis in patients with polycythemia vera: evidence for aspirin-suppressible platelet activation in vivo. *Blood* 1992; 80: 1965–1971.
74 Rao AK, Koike K, Willis J et al. Platelet secretion defect associated with impaired liberation of arachidonic acid and normal myosin light chain phosphorylation. *Blood* 1984; 64: 914–921.
75 Matijevic-Aleksic N, McPhedran P, Wu KK. Bleeding disorder due to platelet prostaglandin H synthase-1 (PGHS-1) deficiency. *Br J Haematol* 1996; 92: 212–217.
76 Dube JN, Drouin J, Aminian M, Plant MH, Laneuville O. Characterization of a partial prostaglandin endoperoxide H synthase-1 deficiency in a patient with a bleeding disorder. *Br J Haematol* 2001; 113: 878–885.
77 Remuzzi G, Benigni A, Dodesini P et al. Reduced platelet thromboxane formation in uremia. Evidence for a functional cyclooxygenase defect. *J Clin Invest* 1983; 71: 762–768.
78 Laffi G, Cominelli F, Ruggiero M, Fedi S, Chiarugi V, Gentilini P. Molecular mechanism underlying impaired platelet responsiveness in liver cirrhosis. *FEBS Lett* 1987; 220: 217–219.
79 Fuse I, Higuchi W, Uesugi Y, Aizawa Y. Pathogenetic analysis of three cases with a bleeding disorder characterized by defective platelet aggregation induced by Ca^{2+} ionophores. *Br J Haematol* 2001; 112: 603–608.
80 Wu G, Wei C, Kulmacz RJ, Osawa Y, Tsai A-L. A mechanistic study of self-inactivation of the peroxidase activity in prostaglandin H synthase-1. *J Biol Chem* 1999; 274: 9231–9237.
81 Jones DA, Fitzpatrick FA. Thromboxane A_2 synthase. Modification during 'suicide' inactivation. *J Biol Chem* 1991; 266: 23510–23514.
82 Marnett LJ, Wright TL, Crews BC, Tannenbaum SR, Morrow JD. Regulation of prostaglandin biosynthesis by nitric oxide is revealed by targeted deletion of inducible nitric-oxide synthase. *J Biol Chem* 2000; 275: 13427–13430.
83 Marcus AJ, Weksler BB, Jaffe EA, Broekman MJ. Synthesis of prostacyclin from platelet-derived endoperoxides by cultured human endothelial cells. *J Clin Invest* 1980; 66: 979–986.
84 Barry OP, FitzGerald GA. Mechanisms of cellular activation by

Cambridge Books Online © Cambridge University Press, 2009

platelet microparticles. *Thromb Haemost* 1999; 82: 794–800.
85 Vane JR, Botting RM. Pharmacodynamic profile of prostacyclin. *Am J Cardiol* 1995; 75: 3A-10A.
86 Murata T, Ushikubi F, Matsuoka T et al. Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature* 1997; 388: 678–682.
87 Ushikubi F, Nakajima M, Hirata M, Okuma M, Fujiwara M, Narumiya S. Purification of the thromboxane A_2/prostaglandin H_2 receptor from human blood platelets. *J Biol Chem* 1989; 264: 16496–16501.
88 Dorn GW 2nd, DeJesus A. Human platelet aggregation and shape change are coupled to separate thromboxane A_2-prostaglandin H_2 receptors. *Am J Physiol* 1991; 260: H327-H334.
89 Hirata M, Hayashi Y, Ushikubi F et al. Cloning and expression of cDNA for a human thromboxane A_2 receptor. *Nature* 1991; 349: 617–620.
90 Thomas DW, Mannon RB, Mannon PJ et al. Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane. *J Clin Invest* 1998; 102: 1994–2000.
91 Raychowdhury MK, Yukawa M, Collins LJ, McGrail SH, Kent KC, Ware JA. Alternative splicing produces a divergent cytoplasmatic tail in the human endothelial thromboxane A_2 receptor. *J Biol Chem* 1994; 269: 19256–19261.
92 Habib A, FitzGerald GA, Maclouf J. Phosphorylation of the thromboxane receptor, the predominant isoform expressed in human platelets. *J Biol Chem* 1999; 274: 2645–2654.
93 Miggin SM, Kinsella BT. Expression and tissue distribution of the mRNAs encoding the human thromboxane A_2 receptor (TP) α and β isoforms. *Biochim Biophys Acta* 1998; 1425: 543–559.
94 Parent JL, Labrecque P, Orsini MJ, Benovic JL. Internalization of the TXA_2 receptor α and β isoforms: role of the differentially spliced COOH terminus in agonist-promoted receptor internalization. *J Biol Chem* 1999; 274: 8941–8948.
95 Vezza R, Habib A, FitzGerald GA. Differential signaling by the thromboxane receptor isoforms via the novel GTP-binding protein, G_h. *J Biol Chem* 1999; 274: 12774–12779.
96 Shenker A, Goldsmith P, Unson CG, Spiegel AM. The G protein coupled to the thromboxane A_2 receptor in human platelets is a member of the novel Gq family. *J Biol Chem* 1991; 266: 9309–9313.
97 Dorn GW 2nd. Tissue- and species-specific differences in ligand binding to thromboxane A_2 receptors. *Am J Physiol* 1991; 261: R145–R153.
98 Hirata T, Kakizuka A, Ushikubi F, Fuse I, Okuma M, Narumiya S. Arg^{60} to Leu mutation of the human thromboxane A_2 receptor in a dominantly inherited bleeding disorder. *J Clin Invest* 1994; 94: 1662–1667.
99 Cayatte AJ, Du Y, Oliver-Krasinki J. The thromboxane receptor antagonist S18886 but not aspirin inhibits atherogenesis in apo E-deficient mice: evidence that eicosanoids other than thromboxane contribute to atherosclerosis. *Arterioscler Thromb Vasc Biol* 2000; 20: 1724–1728.
100 Patrono C, FitzGerald GA. Isoprostanes: potential markers of oxidant stress in atherothrombotic disease. *Arterioscler Thromb Vasc Biol* 1997; 17: 2309–2315.
101 Audoly LP, Rocca B, Fabre JE et al. Cardiovascular responses to the isoprostanes iPF(2alpha)-III and iPE(2)-III are mediated via the thromboxane A(2) receptor in vivo. *Circulation* 2000; 101: 2833–2840.
102 Cipollone F, Patrignani P, Greco A et al. Differential suppression of thromboxane biosynthesis by indobufen and aspirin in patients with unstable angina. *Circulation* 1997; 96: 1109–1116.
103 Cimetiere B, Dubuffet T, Muller O et al. Synthesis and biological evaluation of new tetrahydronaphthalene derivatives as thromboxane receptor antagonists. *Bioorg Med Chem Lett* 1998; 8: 1375–1380.
104 FitzGerald GA, Patrono C. The anti-inflammatory properties and safety of coxibs, selective inhibitors of cyclooxygenase-2. *N Eng J Med* (In press).

Cambridge Books Online © Cambridge University Press, 2009

Platelet procoagulant activities: the amplification loops between platelets and the plasmatic clotting system

H. Cœnraad Hemker

Synapse b.v.; Cardiovascular Research Institute Maastricht, Maastricht, The Netherlands

Introduction

Before the early 1960s interaction between platelets and plasmatic thrombin or 'thromboplastin' generation was the subject of intensive study[1]. Since the introduction of the aggregation and adhesion methods, most studies on platelets are done under conditions where clotting is prevented, whereas blood coagulation is usually studied in the absence of platelets. In this way only indirect information (e.g. recognition of thrombin-receptors) is obtained on the interaction between platelet and plasma. Direct information on the platelet-clotting system reinforcement loops has been reappearing only recently.

In the body, platelet activation and thrombin generation are two intimately linked processes. Thrombin is a potent platelet activator and adequate thrombin formation is impossible without activated platelets. Because thrombin generation and platelet activation are mutually interdependent, each process requiring the product of the other for its full activity, the two are interlocked in positive feedback. Unlike the better known negative feedback that has regulatory properties, a positive feedback loop amplifies product formation explosively. Hemostasis is an intricate complex of positive and negative feedback loops. Here, we will focus on the amplification mechanism constituted by the platelet–clotting system interaction. The thrombin induced positive feedback loop has been known for over half a century (see fig. 26.1 from ref.1) but only recently has it again become a focus of wider interest.

Two questions are essential to the understanding of this loop: (a) what is the nature of the procoagulant activity of activated platelets? and (b) how do the products of the clotting process, i.e. thrombin and fibrin, bring about procoagulant activity in platelets?

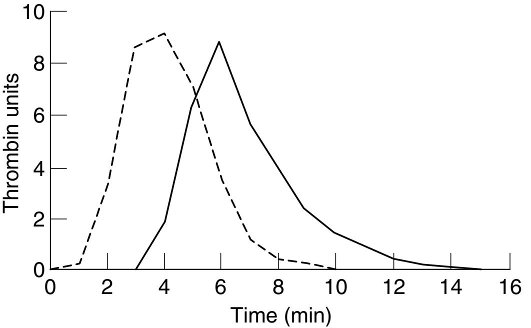


Fig. 26.1. Thrombin mediated feedback anno 1953. From ref. 1, p.97. The original legend reads: *The thrombin generation test was carried out on normal whole blood in glass tubes with and without the addition of thrombin to the blood. The presence of thrombin greatly shortens the delay phase before rapid thrombin formation occurs.*

Adhesion and aggregation are early events and make us focus on the start of plug formation. However, the role of platelets is not finished with the forming of an aggregate adhering to a lesion. Neither does clot formation mark the end of the activity of the clotting factor system. Over 97% of all thrombin appears after a clot has formed, in a mechanism that is critically dependent upon platelet function[2]. Such thrombin, among other things, determines the resistance of the clot to fibrinolysis[3] and the extent of the thrombotic reaction around a primary focus, brought about by the numerous actions of thrombin on the cells of the blood and the vessel wall. Clot retraction is another example of a late physiological reaction that is entirely dependent on platelets in a clot.

The importance of such late processes becomes apparent from clinical observation. Resistance to activated

protein C or heparin administration hardly influence the start of the haemostatic/thrombotic reaction triggered by tissue factor (TF). Neither are bleeding times or TF induced clotting times significantly influenced. The clinically observed pro- or antithrombotic state does, however, correlate well with the changes in the amount of thrombin formed after TF induced clotting.

Essentials of thrombin formation

When clotting is activated in blood or plasma, a dynamic situation builds up in which prothrombin is converted into thrombin and thrombin disappears by its interaction with antithrombins. Clotting occurs at the very beginning of the process, as soon as 5–10 nM of thrombin are formed. The peak of thrombin concentration is seen a few minutes later and, depending upon the reaction mixture, amounts to 50–250 nM. Normally only ~10% of all prothrombin remains unconverted.

The prothrombin converting complex (prothrombinase) consists of factor Xa and factor Va reversibly bound to the surface of a membrane containing coagulant phospholipids (CPL). Negatively charged or aminophospholipids, i.e. phosphatidylserine (PS) and/or phosphatidylethanolamine (PE) are essential components of CPL[4]. The factor X activating enzyme is either a complex of factor VIIa and (membrane bound) TF or it is 'tenase', a complex analogous to prothrombinase with factors IXa and VIIIa in the role of factors Xa and Va, respectively[5]. In prothrombinase, factor Va increases the rate of thrombin formation a thousand fold[6,7]. Factor Va is formed from plasmatic factor V by the action of (meizo-)thrombin[8]. The first traces of (meizo-) thrombin formed therefore tremendously increase thrombin formation. (Meizothrombin is a prothrombin activation intermediate that has retained the membrane binding properties of prothrombin but already has the proteolytic activities of thrombin). Thrombin also is essential in the activation of protein C and therefore entails factor Va breakdown in a later stage. Factor V activation and subsequent inactivation in this manner create a limited time lapse to which thrombin generation is confined.

The binding of prothrombinase and its substrate prothrombin (factor II) to CPL makes the proteins meet in the required juxtaposition and increases the local concentration of substrate around the enzyme, which kinetically translates into an upto one thousand fold decrease of Km. Not only the composition but also the size of the available surface influences the kinetic properties of the enzymes. A prothrombinase complex on a large CPL surface is more efficient (K_m decreases more) than that on a small aminophospholipid containing vesicle because the surface around the complex acts as a 'funnel' that guides prothrombin from free solution to the enzyme through lateral diffusion[9]. For the interpretation of experiments on clotting factor-platelet interaction it is essential to realise that mere changes in lipid availability and composition, as they occur during platelet activation, may profoundly change the binding and kinetic characteristics of the platelet membrane. This may be one of the reasons that a number of so far elusive clotting factor receptors have been postulated (see below).

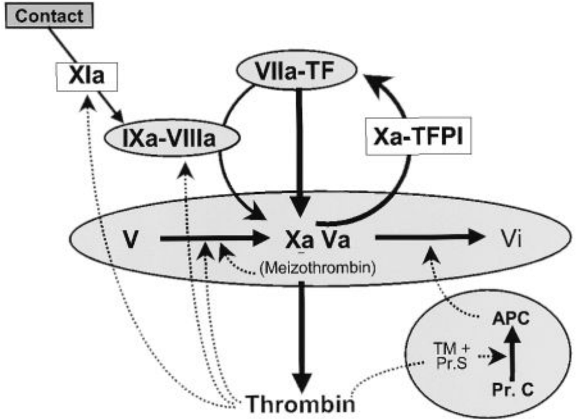


Fig. 26.2. A Coagulation Scheme for 2001. The main vertical axis is the cascade that ensures thrombin formation by sequential activation. The horizontal axis is the regulation dimension operating through activation of factor V (and VIII and XI) and subsequent inactivation of Va (and VIIIa) by APC.
TF: Tissue factor; TFPI: Tissue factor pathway inhibitor; TM: thrombomodulin; Pr.S: protein S; Pr.C: protein C; APC: activated protein C. suffix a: activated form, suffix i: inactivated form. The arrows indicate either chemical conversion sequential activation and the stippled ones, enzymatic activity. TFPI reacts stoichiometrically with Xa and the complex inactivates the TF-VIIa complex by binding to it.

As stated above, factor Xa is formed from factor X either by the TF – factor VIIa complex (the so called 'extrinsic' pathway) or by 'tenase' formed from factors IXa and VIIIa. Factor IXa is formed in the body by TF-VIIa and by (thrombin-activated) factor XIa. In the lab it forms via contact activation, notably during the determination of the activated partial thromboplastin time (aPTT), where a contact activator and CPL are added together with Ca^{2+} to start the so called 'intrinsic' reaction pathway. The clotting time in this case is the lag-time required for traces of thrombin to activate factor VIII that, in plasma, is bound to von Willebrand factor (vWF) and therefore, unlike factor V, will

Cambridge Books Online © Cambridge University Press, 2009

not adsorb upon the CPL surface before it is activated and hence cannot be activated by membrane-bound meizothrombin. It remains anybodies guess how far the situation changes when vWF, by binding to platelet receptors, brings factor VIII close to the platelet surface (see below).

TF occurs abundantly on perivascular connective tissue cells. These will express CPL only in so far as they are damaged. In a wound, contact establishes between TF and factor VII(a) from plasma. TF does not normally occur on circulating platelets but it is deposited on adherent platelets by leukocytes[10] (see below). It can thus be imagined that on damaged cells and platelets provided with TF the complete thrombin generation process can occur on the same surface. This is probably not the rule, however, otherwise the antithrombin–pentasaccharide complex, which acts on free factor Xa only, would not have an antithrombotic effect[11].

Means to study clotting factor – platelet interaction

The procoagulant properties of isolated platelets in suspension can be studied by measuring the conversion of added prothrombin and manipulating the concentration of the constituents of prothrombinase so that the one to be studied is rate limiting. The appearance of factor V(a) or CPL or other prothrombinase sustaining activity can thus be assessed. The property of Annexin V to bind to CPL only, can be conveniently used to inhibit CPL dependent processes or to spot CPL on cells in FACS-experiments[12,13]. On adherent platelets similar activity measurements in flow chambers can be combined with direct observation of morphological changes. The appearance of CPL can be observed directly by the binding of fluorescently labelled annexin V as well as the measurement of intracellular Ca^{2+} after loading the cells with a dye, the fluorescence of which is dependent upon the Ca^{2+} concentration.

In 1989 Béguin reintroduced measurement of thrombin generation (TG) in PRP[2]. Classical TG-experiments require inconvenient, frequent subsampling from recalcified PRP. Automated registration of the TG-curve with a fluorescent thrombin substrate, i.e. the thrombogram, circumvents this problem[14] (Fig. 26.3). In this type of experiment, the platelet-plus-plasma system is left intact as far as possible. It therefore allows direct investigation of their interaction under near to physiological conditions.

The appearance rate of thrombin is dependent upon the amount of prothrombin and on the activity of prothrombinase. The disappearance rate is proportional to the activity of the antithrombin present. This allows us to calculate the rate of conversion of prothrombin into thrombin[15]. At normal prothrombin- and antithrombin concentration and in the absence of heparin (-likes) that boost antithrombin action, this calculation is not necessary because the course of thrombin concentration directly reflects prothrombin conversion. So, in experiments such as those of Fig. 26.3 the differences between the thrombograms reflect the differences in the availability of the rate limiting component of prothrombinase.

In the extrinsic system thrombin formation immediately starts. In the intrinsic system and in PRP, thrombin generation is under normal detection limits for some time (= lag-time). Due to the fact that clotting occurs very early after the start of massive thrombin generation, this lag-time for all practical purposes equals the clotting time.

If no platelets are present (PPP), thrombin generation requires added CPL as well as Ca^{2+}. The trigger can be either TF or a contact activator (e.g. kaolin, ellagic acid). If the PPP is devoid of platelet remnants and a trigger is omitted, it may take an hour before thrombin will form after mere recalcification. Contrary to PPP, recalcification makes PRP clot without further triggering, after a lag-time that is dependent upon the degree of damage done to the platelet in obtaining the PRP. If the PRP is tested within one hour after a flawless venipuncture without suction and avoiding exposure to temperatures below 15 °C, the lag-time is 9 ± 2 min. The subtle damage brought about by washing or gel filtering already may reduce the lag time by several minutes. The closest approach to physiological conditions remains the study of blood directly from a venipuncture through a flow chamber[10]. The drawback is that, for all practical purposes, the observations remain restricted to histological and histochemical observation.

The role of platelets in thrombin formation

Because platelets stick to collagen and fibrin and expose their procoagulant properties after activation, they serve to localise thrombin generation in a wound or thrombus (Fig. 26.4). The enhancement of thrombin generation due to platelet collagen-interaction, acts via the receptors integrin $\alpha2\beta1$ and GP-VI[16,17]; see further[18].

Four types of procoagulant activity have been described for activated platelets: (a) release of clotting factors; (b) appearance of CPL; (c) activation of clotting factor receptors, and (d) a role in exposing TF from white blood cells.

(*a*) Activated platelets release factor V and von Willebrand factor (vWF) as well as fibrinogen[19–22]. Factor V is shed in unactivated form and activated by thrombin afterwards[23]. The physiological function of factor V release

Cambridge Books Online © Cambridge University Press, 2009

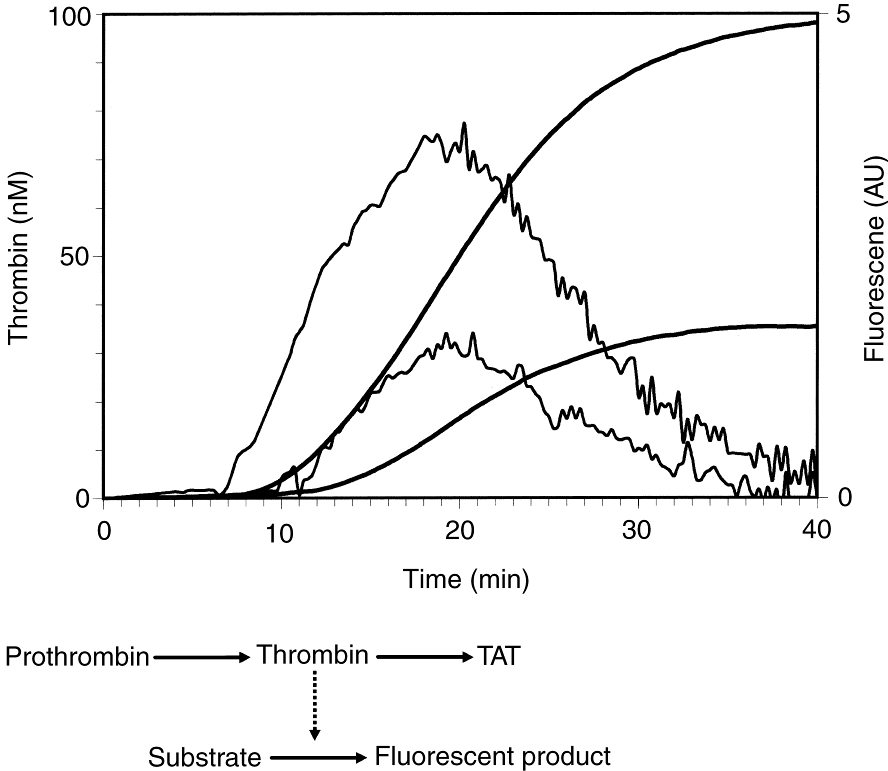


Fig. 26.3. The Thrombogram. Continuous measurement of thrombin generation, using a fluorescent signal and a substrate that is not split by α2Macroglobulin-bound thrombin. Thick lines: fluorescent signal. Thin lines: first derivative. Upper lines: normal PRP; lower lines: PRP with 20 µg/ml abciximab added.

is difficult to understand in terms of thrombin generation in plasma because, as is already known from the earliest studies on factor V, 10% or less of the normal plasma concentration of this factor is required for nearly normal thrombin generation. One can surmise that release serves a purpose for thrombin generation within a platelet aggregate. Adhesion and aggregation are the first steps in haemostasis but thrombin generation follows almost immediately and no stable thrombus or haemostatic plug will arise without it. The amount of plasma factors entrapped in the interstices of the aggregate is necessarily limited. In plasma, factor X is in 6–8 fold excess over factor V. For rapid and adequate thrombin formation it may therefore be required that more factor V is supplied via the release reaction. Recent results indeed suggest strongly that released factor V is preferably bound to the surface of procoagulant platelets[24].

Also factor IX, in plasma, is in excess over factor VIII (over 50-fold). There is no compelling evidence that factor VIII is released from platelets. This factor, however, is carried by vWF, the binding of which to GPIb and activated $\alpha_{IIb}\beta_3$ on the surface of platelets is well documented. Binding of vWF concentrates factor VIII near the platelet surface. As soon as some thrombin is generated on the platelet surface, this will activate this factor VIII. By making elegant use of the fact that human factor VIII binds to mouse vWF but human vWF hardly to mouse platelets, it could be shown that binding of factor VIII to the thrombus indeed has a prothrombotic effect[25]. This is corroborated by the observation that prothrombin consumption is decreased in patients with a congenital lack of GPIb and that this defect is corrected by addition of factor VIII[26]. This mechanism may explain the correlation between the level of factor VIII and thrombosis found in epidemiological studies[25].

Normally, during the clotting process, all fibrin is formed before 3% of prothrombin is converted but almost the totality of prothrombin available is consumed due to ongoing thrombin formation in the clot. As discussed in the introduction, this ‘excess’ amount of thrombin is an important determinant of the extent of the thrombotic/haemostatic reaction. Under low flow conditions, the thrombin generated in the aggregate/clot will act on

Cambridge Books Online © Cambridge University Press, 2009

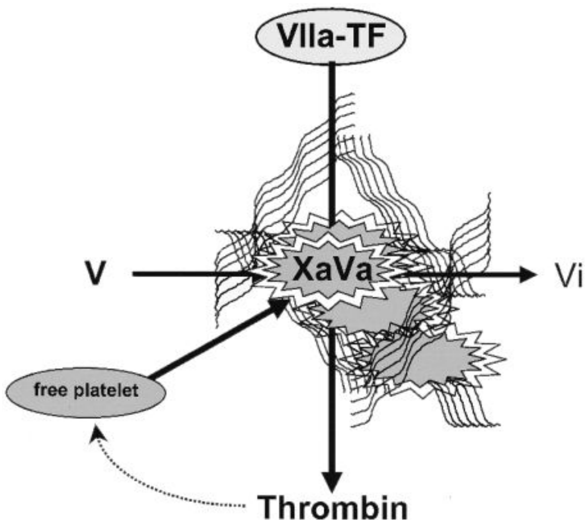


Fig. 26.4. The three-dimensions of thrombin generation. The first dimension (Y) is thrombin production through the cascade mechanism. The second one (X) ensures limitation in time through thrombin-governed positive and negative feedback. The third (Z) localizes thrombin generation on collagen or fibrin fibres through adhesion and aggregation and exposure of platelet CPL through these fibres and thrombin.

the surrounding blood and make it clot. At the fibrin–blood interface of that new clot more platelets will adhere (see below for the link: fibrin – vWF – GPIb – platelet) and become procoagulant, which starts the process again and makes the thrombus grow. The alternation of blood clot and platelet aggregates explains the striated aspect often observed in venous thrombi. Under high flow conditions thrombin that diffuses out of the aggregate/clot will be strongly diluted and washed away. Only a few fibrin fibres are to be found at the outside[27] and no erythrocytes will be entrapped. On this fibrin, to which active thrombin remains bound, new platelets may adhere and become procoagulant in their turn, by two mechanisms[28]: (*a*)activation by fibrin-bound thrombin, and (*b*) activation by fibrin-bound vWF, as discussed further.

(*a*) The amount of thrombin formed in a clot also determines the degree of activation of TAFI and therefore, indirectly, the resistance of the clot against fibrinolysis[3,29,30]. This may well explain the clinical observation that in deficiencies of factors FVIII, IX and XI loose clots form that are subject to easy lysis.

(*b*) Studies from the van Deenen group in the early 1970s had demonstrated that the phospholipid components of the cell membrane are not evenly distributed between the inner and the outer leaflet. PS and PE, essential for CPL-function, are to be found at the inside[31–35]. Therefore a cell membrane is not procoagulant unless wounded, apoptotic or otherwise disturbed (e.g. by hemoglobin crystals in depranocytosis). Platelets are the only known cells in which the loss of the normal asymmetry ('flip-flop') is induced as a part of their physiological function. In this way platelet activation brings about their power to facilitate blood coagulation[36]. How far the process is similar to membrane scrambling in apoptosis remains to be investigated.

Plateletmembranes scramble as a last stage in their activation after shape change, aggregation and release. This stage is only attained when they are activated by thrombin, by collagen and, as we will see later, by fibrin and vWF. Epinephrine, ADP, prostanoids etc. may modulate the scrambling response but will not, by themselves, bring it about.

The intra-membrane activity responsible for the break of asymmetry is attributed to three putative mechanisms (i) an inward-directed pump ('flippase') for PS and PE; (ii) an outward-directed pump ('floppase') and (iii) a lipid scramblase, which facilitates bidirectional migration across the bilayer of all phospholipid classes[37]. These activities await further description and definition in terms of protein entities. The precise intracellular events that activate the intramembrane mechanism remain largely obscure. It is clear that a rise in intracellular Ca^{2+} is required: any Ca^{2+}-ionophore is a potent inducer of flip-flop[18]. In the next section we will describe which platelet receptors may be involved in triggering the process.

Platelet activation goes hand in hand with extensive rearrangement of the cytoskeleton. In this process patches of the membrane bulge outward and pinch off ('blebbing')[38]. This causes minute vesicles (microparticles) to appear, the CPL activity of which can be demonstrated (e.g. by annexin V binding) and the platelet origin of which is clear from their containing integral platelet membrane proteins such as $\alpha_{IIb}\beta_3$ and P-selectin[39,40]. Calpain inhibitors, that inhibit cytoskeleton degradation[41] also inhibit blebbing and microparticle formation as well as thrombin generation in PRP (unpublished results). The microparticles can be demonstrated in the serum left after a thrombin generation experiment and their appearance is inhibited by antibodies against vWF, GPIb and against $\alpha_{IIb}\beta_3$; in the presence of fibrin, antibodies against GPIb/IX also inhibit (Keularts, publication in preparation). Microparticles bind to fibrin[42], so at least part of them remain normally in the emerging thrombus. It is not known whether they are shed in the bloodstream, but they have been found in the peripheral circulation in various pathological conditions[43–49].

(*c*) A number of receptors have been described that bind

Cambridge Books Online © Cambridge University Press, 2009

clotting factors to the platelet membrane. Confusingly, high affinity binding sites for clotting factors that arise from the exposure of CPL are sometimes called receptors. Here, we define receptors in the strict sense, as integral platelet membrane proteins that engage in a protein-protein interaction with clotting factors. Such receptors have been postulated for all known factors and rigorously demonstrated only for thrombin (see later).

Prothrombin

Zybacova and Plow have described that $\alpha_{IIb}\beta_3$ can act as a receptor for prothrombin[50]. A binding site that is shared with factor X has also been described[51].

Factor V(a)

Platelet receptor-dependent binding of factors Va and VIIIa has been described[52].

Factor X(a)

EPR-1, a receptor found and characterized on fibroblasts[53], is presumed to coordinate assembly of the prothrombinase complex. The site shared with prothrombin is described to have different properties[51].

Factor VIII(a)

A factor VIIIa receptor has been described[54] and it has also been surmised that GPIb, by virtue of its binding vWF, may serve to bring factor VIII in the vicinity of the platelet membrane[26]. It has been demonstrated that vWF binds to fibrin[55] and to the platelet surface in a thrombus and that this process leads to enrichment in factor VIII[25]. So vWF binding causes concentration of factor VIII near the platelet membrane.

Factor IX(a)

A factor IXa receptor on activated platelets has been postulated, that facilitates factor X activation[56–58].

Factor XI(a)

Through its apple 3 domain factor XI binds to a few high affinity sites on activated platelets[59] but also via high molecular weight kininogen.[60] The former facilitates FXI activation by thrombin on activated platelets[61,62]. In contrast, in fresh plasma from severely factor XI deficient patients, we found an essentially identical relation between the concentration of added factor XI and thrombin generation in PRP, in PPP supplemented with normal platelets and in PPP supplemented with 1 μM of synthetic CPL (Keularts, Zivellin, Béguin, Seligsohn and Hemker. Thrombosis Haemostasis, in press 2001), i.e. we found no evidence for a facilitating role of platelets in thrombin generation when factor XI is rate limiting.

Except for EPR-1 no specific protein receptors have been identified. In our opinion the evidence produced for the existence of most or all of these receptors is open for alternative interpretation. The observations might, for example, be brought about by changes in the lipid composition of the outer face of the platelet membrane. During platelet activation the composition and the size of the available CPL and therefore the kinetic characteristics of clotting factor binding and conversion increase considerably. This can mimic the effect of specific receptors. It may be extremely difficult to distinguish between a protein receptor for a clotting factor and a receptor that is instrumental in producing CPL with high affinity for a clotting factor.

The kinetic effects of platelets and of phospholipids extracted from platelets are of the same magnitude. Rigorous experimental proof would require the demonstration of thrombin formation in the absence of CPL, either by isolating the putative receptor to purity or by inhibiting CPL-dependent clotting factor activation completely. Such evidence to our knowledge has not been brought forward. As long as solid proof to the contrary has not been presented, we prefer not to multiply hypotheses beyond necessity and continue to explain the procoagulant phenomena on the surface of activated platelets in terms of CPL.

(*d*) When blood is led directly from a vein through a flow chamber in which collagen is exposed (either arterial media or purified collagen on glass), platelets adhere to the collagen. Such platelets pick up TF from leukocytes that roll over the surface of the aggregate; this TF is biologically active. In the free platelet no TF can be demonstrated. On the surface of the free leukocytes there is no active TF either, so TF is 'decrypted' in the process. The specific leukocyte membrane marker CD15, absent in circulating platelets, is present in adherent platelets that have been exposed to leukocytes, which shows that the leukocyte–platelet contact involves the carryover of pieces of leukocyte membrane. This process, together with the flip-flop induced by fibrin and by fibrin-bound thrombin, makes it possible that a plug or thrombus grows even though the collagen and perivascular TF-carrying cells are shielded from contact with flowing blood by the emerging thrombus/plug[10].

Which type of leukocyte is involved in these processes is still a matter of debate. Giesen et al. suggest that polymorphonuclear cells as well as monocytes contribute.

Cambridge Books Online © Cambridge University Press, 2009

Østerud et al.[63], in experiments in which TF synthesis is induced by lipopolysaccharides, conclude that only monocytes are able to synthesize TF but that platelets as well as granulocytes play a supporting role.

The role of thrombin and fibrin in provoking platelet procoagulant activity

The early effect of thrombin on the procoagulant capacity of platelets is triggering release (and subsequent activation) of, among many other substances, clotting factor V. At a later stage the platelet surface acquires procoagulant activities. The release reaction (see Chapter 24) can be provoked by a number of agents. The exposure of CPL is brought about by thrombin, collagen and vWF-fibrin only.

After recalcification of PRP, the burst of thrombin generation is preceded by the appearance of subnanomolar amounts (<0.1 U/ml) of thrombin, probably formed on traces of circulating procoagulant phospholipid or an occasional damaged platelet, possibly started by minute amounts of circulating TF or minimal contact activation. Adding such amounts of thrombin at zero time dramatically shortens the lag-time as already observed by Biggs and Macfarlane[1] (see Fig. 26.1) and similar amounts of a specific thrombin inhibitor like hirudin prolong it[2]. The lag phase can also be shortened by addition of synthetic CPL, through membrane scrambling by a Ca^{2+}- ionophore or by freeze–thawing. On the other hand, adding factor VIIIa does not abolish the lag phase unless CPL is available first. From these and similar observations we conclude that, in PRP, the availability of CPL is the rate-limiting component for thrombin generation and that thrombin or a thrombin product causes the platelets to produce it.

Which receptors are activated by thrombin may depend upon the amount of thrombin present. In PRP it may therefore be different during the lag-phase and thereafter. In washed platelets it has been shown[64] that blocking the PAR-1 receptor with a peptide (YFLLRNP), with bradykinin or with an antibody, blocks the appearance of CPL. Thrombin activating peptide (SFLLRN), although causing aggregation, does not provoke CPL but rather renders platelets insensitive to thrombin. Dörmann et al. also reported that CPL production in washed platelets is inhibited by antibodies against the thrombin binding site in GPIb and by glycocalicin. Unlike α-thrombin, γ-thrombin, which lacks the normal anionic exosite of α-thrombin, does not provoke CPL. These observations indicate a crucial involvement of thrombin-GPIb interaction and in all probability both PAR-1 and GPIb play a role.

Thrombin contact induces the activation of $\alpha_{IIb}\beta_3$ complex in the platelet membrane. Adding an antibody (abciximab) or another antagonist (MK383) to PRP partially inhibits thrombin generation[65–67,68]. So $\alpha_{IIb}\beta_3$ is part of a loop consisting of the sequence: thrombin $\rightarrow$ GPIb and/or PAR $\rightarrow$ $\alpha_{IIb}\beta_3$ $\rightarrow$ CPL $\rightarrow$ prothrombinase $\rightarrow$ thrombin. Inhibition via blocking $\alpha_{IIb}\beta_3$ is not complete, so the loop described is not likely to be the only pathway. If no fibrin(-ogen) is present, antibodies against GPIb will not inhibit thrombin generation but antibodies against vWF do[28,69,70]. This shows that (a) in PRP GPIb is not the only thrombin receptor involved and (b) vWF–$\alpha_{IIb}\beta_3$ interaction must play a role.

The addition of a fibrin clot after recalcification of PRP considerably shortens the lag phase[69]. This is partly due to fibrin bound thrombin that is able to activate platelets and other clotting factors. Also several other serum proteins (e.g. factor Xa) and platelet remnants[42] may be incorporated in a natural clot. When, however, purified fibrinogen is clotted with a snake venom enzyme that as such does not activate platelets, the pure fibrin clot that is obtained will still provoke platelet procoagulant activity[28], notably if $\alpha_{IIb}\beta_3$ is also blocked. This direct activation by fibrin can be observed via thrombin generation experiments but also in EM pictures, if care is taken to prevent thrombin playing a role (Fig. 26.5). Platelet–fibrin interaction can be inhibited by antibodies against GPIb and/or vWF. Preventing fibrin polymerization or removing fibrin as soon as it forms blocks this pathway. Our current hypothesis is that vWF adsorbs onto fibrin and thus undergoes the conformational change that makes it a ligand for GPIb. This constitutes a second feedback activation loop: thrombin $\rightarrow$ fibrin $\rightarrow$ fibrin-vWF $\rightarrow$ GPIb $\rightarrow$ CPL $\rightarrow$ prothrombinase $\rightarrow$ thrombin.

Because of the loop character of these sequences we cannot, in thrombin generation experiments, discern between the above sequence and a nested loop in which, e.g. thrombin and GPIb together are required for CPL formation. Experiments with platelets adherent to fibrin are in favour of this possibility (Briedé, manuscript in preparation). On the other hand, the platelet activation on fibrin is seen in EM pictures in which the possibility of thrombin generation was practically excluded (Fig. 26.5) and the polymerization of fibrinogen brought about by an otherwise inert snake venom enzyme enhances thrombin generation through GPIb-dependent platelet activation (unpublished results).

The fibrin–vWF–GPIb pathway of platelet activation remains active when $\alpha_{IIb}\beta_3$ is blocked with an antibody. The $\alpha_{IIb}\beta_3$ pathway remains active when GPIb is blocked or in the absence of polymerized fibrin. The two receptors

Cambridge Books Online © Cambridge University Press, 2009

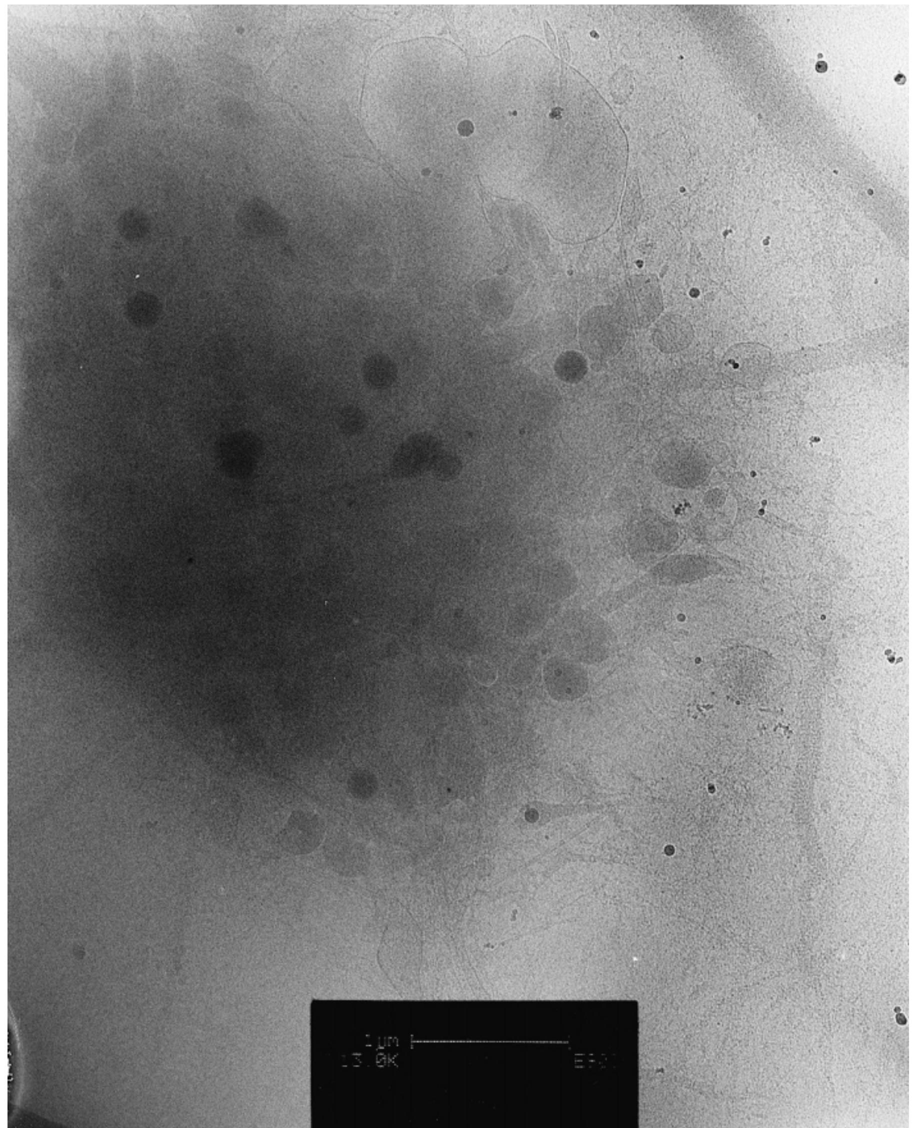


Fig. 26.5. Activated platelets on a fibrin mesh.
On a grid a fibrin mesh was made by clotting pure fibrinogen with a snake venom enzyme. Freshly drawn blood was collected on a mixture of hirudin (2 μM) and 0.5 mM of PPACK (both thrombin inhibitors). The grid was held in the anticoagulated PRP for 1 minute, washed in a saline solution, snap frozen and mounted for observation directly.

therefore seem to function on parallel pathways and both pathways are inhibited by lack of vWF or by vWF antibodies. Also in platelets adherent on fibrinogen, vWF has been shown to be required for CPL exposure[71].

The findings in PRP and with gel-filtered platelets are in accordance as to the involvement of the receptors GPIb and $\alpha_{IIb}\beta_3$[64]. Whereas the inhibition obtained by blocking these receptors in washed platelets is >90%, those in PRP do not exceed 50% which suggests that they are coupled in series in one condition and on parallel pathways in the other[70,71]. Also, in PRP, vWF is a compulsory factor but not in washed platelets. How these apparently contradictory observations may be reconciled is not clear at the moment. It must be kept in mind that the experimental conditions are significantly different, e.g. in the experiments with washed platelets fibrin polymerization is inhibited, which might prevent activation of vWF. The necessity to add polymerization inhibitors suggests that, at some stage, traces of thrombin arise.

Anyhow, in patients who lack vWF, thrombin generation

Cambridge Books Online © Cambridge University Press, 2009

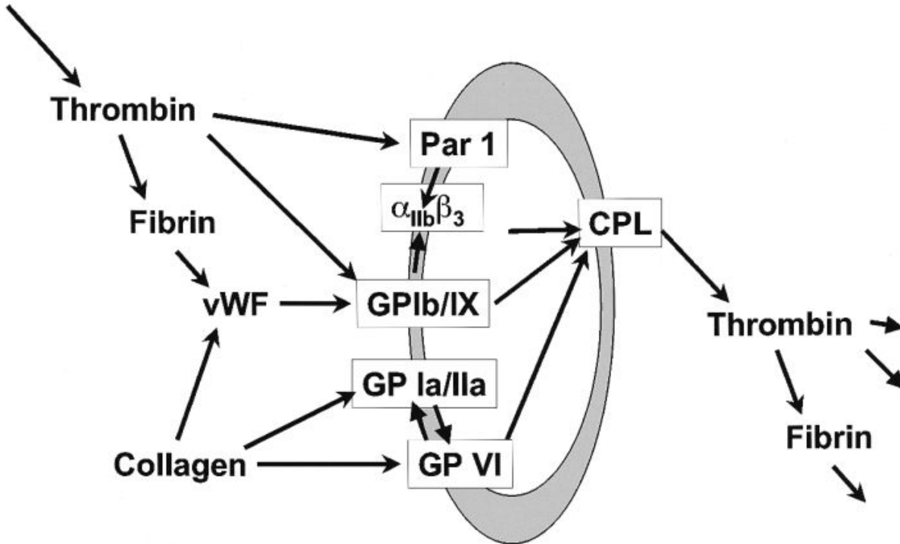


Fig. 26.6. The perpetuum model of thrombus formation. The arrows indicate activations, independent of the mechanism involved: binding, proteolysis or more complicated intraplatelet mechanisms.

in PRP is severely diminished, in a process that cannot be attributed to a lack of factor VIII[72]. Also, thrombin generation can be blocked over 75% with antibodies against vWF and such antibodies have an additional inhibitory power in the presence of antibodies against either of the two GPs involved (Keularts et al. publication in preparation). Under these conditions, and therefore presumably in vivo, vWF must play a role in both the $\alpha_{IIb}\beta_3$ and the GPIb dependent pathway. vWF, in PRP, is a clotting factor, in the sense that its presence is required for the generation of thrombin. This is explicitly not due to the fact that vWF carries factor VIII because the defect is not seen if sufficient CPL is present, i.e. in contact activated PPP with added CPL or in PRP where the platelet membrane is scrambled with a Ca^{2+}-ionophore.

In agreement with this proposed mechanism, Glanzmann's thrombasthenia as well as a- and hypofibrinogenemia and vWF deficiency cause a decreased thrombin generation in PRP (Fig. 26.6). Thrombin generation in PPP is normal in Glanzmann's disease and slightly increased in afibrinogenemia. In vWF disease thrombin-generation in PPP (intrinsic system) is normal as long as the factor VIII level is not below ~10%. Half normal thrombin generation in PRP is seen at a vWF level of ~40% (F.VIII 100%) and a F.VIII level of ~5% (vWF 100%)[73].

In conclusion, in PRP, the interaction between the plasmatic coagulation system and the platelet is close and mutually reinforcing. A minimal hypothesis requires two parallel, receptor dependent pathways for the appearance of CPL, that both need vWF: (a) via the $\alpha_{IIb}\beta_3$ heterodimer that originates upon the appearance of subnanomolar traces of thrombin; (b) via GPIb that interacts with vWF adsorbed onto formed fibrin. In the presence of collagen a third pathway, involving GP VI and $\alpha 2\beta 1$, plays a role.

References

1 Biggs R, Macfarlane RG. *Human blood coagulation and its disorders.* Blackwell Scientific Publications, Oxford; 1953.

2 Beguin S, Lindhout T, Hemker HC. The effect of trace amounts of tissue factor on thrombin generation in platelet rich plasma, its inhibition by heparin. *Thromb Haemost* 1989; 61: 25–29.

3 Von dem Borne PA, Bajzar L, Meijers JC, Nesheim ME, Bouma BN. Thrombin-mediated activation of factor XI results in a thrombin- activatable fibrinolysis inhibitor-dependent inhibition of fibrinolysis. *J Clin Invest* 1997; 99: 2323–2327.

4 Hemker HC, Esnouf MP, Hemker P W, Swart AC, Macfarlane RG. Formation of prothrombin converting activity. *Nature* 1967; 215: 248–251.

5 Hemker HC, Kahn MJ. Reaction sequence of blood coagulation. *Nature* 1967; 215: 1201–1202.

6 Nesheim ME, Taswell JB, Mann KG. The contribution of bovine Factor V and Factor Va to the activity of prothrombinase. *J Biol Chem* 1979; 254: 10952–10962.

7 Rosing J, Tans G, Govers-Riemslag JW, Zwaal RF, Hemker HC. The role of phospholipids and factor Va in the prothrombinase complex. *J Biol Chem* 1980; 255: 274–283.

8 Tans G, Nicolaes GA, Thomassen MC et al. Activation of

Cambridge Books Online © Cambridge University Press, 2009

human factor V by meizothrombin. *J Biol Chem* 1994; 269: 15969–15972.

9 Giesen PL, Willems GM, Hemker HC, Hermens WT. Membrane-mediated assembly of the prothrombinase complex. *J Biol Chem* 1991; 266: 18720–18725.

10 Giesen PLA, Rauch U, Bohrman B et al. Blood-borne tissue factor, another view of thrombosis. *Proc Natl Acad Sci USA* 1999; 96: 2311–2315.

11 Hemker HC, Choay J, Beguin S. Free factor Xa is on the main pathway of thrombin generation in clotting plasma. *Biochim Biophys Acta* 1989; 992: 409–411.

12 van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, Reutelingsperger CP. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* 1998; 31: 1–9.

13 Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* 1994; 84: 1415–1420.

14 Hemker HC, Giesen PL, Ramjee M, Wagenvoord R, Beguin S. The thrombogram: monitoring thrombin generation in platelet rich plasma. *Thromb Haemost* 2000; 83: 589–591.

15 Hemker HC, Willems GM, Beguin S. A computer assisted method to obtain the prothrombin activation velocity in whole plasma independent of thrombin decay processes. *Thromb Haemost* 1986; 56: 9–17.

16 Siljander P, Farndale RW, Feijge MA et al. Platelet adhesion enhances glycoprotein VI-dependent procoagulant response: involvement of p38 MAP kinase and calpain. *Arterioscler Thromb* (in press) 2001;

17 Heemskerk JW, Siljander P, Vuist WM et al. Function of glycoprotein VI and integrin alpha2beta1 in the procoagulant response of single, collagen-adherent platelets. *Thromb Haemost* 1999; 81: 782–792.

18 Heemskerk JW, Siljander PR, Bevers EM, Farndale RW, Lindhout T. Receptors and signalling mechanisms in the procoagulant response of platelets [In Process Citation]. *Platelets* 2000; 11: 301–306.

19 Sultan Y, Maisonneuve P, Angles-Cano E. Release of VIII R: Ag and VIII R: WF during thrombin and collagen induced aggregation. *Thromb Res* 1979; 15: 415–425.

20 Koutts J, Walsh PN, Plow EF, Fenton JWd, Bouma BN, Zimmerman TS. Active release of human platelet factor VIII-related antigen by adenosine diphosphate, collagen, and thrombin. *J Clin Invest* 1978; 62: 1255–1263.

21 Sixma JJ, Akkerman JW, van Oost B, Gorter G. Intracellular localization of fibrinogen in human blood platelets. *Bibl Haematol* 1977; 44: 129–133.

22 Osterud B, Rapaport SI, Lavine KK. Factor V activity of platelets: evidence for an activated factor V molecule and for a platelet activator. *Blood* 1977; 49: 819–834.

23 Baruch D, Hemker HC, Lindhout T. Kinetics of thrombin-induced release and activation of platelet factor V. *Eur J Biochem* 1986; 154: 213–218.

24 Alberio L, Safa O, Clemetson KJ, Esmon CT, Dale GL. Surface expression and functional characterization of alpha-granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin. *Blood* 2000; 95: 1694–1702.

25 Kawasaki T, Kaida T, Arnout J, Vermylen J, Hoylaerts MF. A new animal model of thrombophilia confirms that high plasma factor VIII levels are thrombogenic. *Thromb Haemost* 1999; 81: 306–311.

26 Bellucci S, Girma JP, Lozano M, Meyer D, Caen JP. Impaired prothrombin consumption in Bernard–Soulier syndrome is corrected in vitro by human factor VIII. *Thromb Haemost* 1997; 77: 383–386.

27 Wester J, Sixma JJ, Geuze JJ, van der Veen J. Morphology of the early hemostasis in human skin wounds: influence of acetylsalicylic acid. *Lab Invest* 1978; 39: 298–311.

28 Kumar R, Beguin S, Hemker HC. The effect of fibrin clots and clot-bound thrombin on the development of platelet procoagulant activity. *Thromb Haemost* 1995; 74: 962–968.

29 Broze GJ, Higuchi DA. Coagulation-dependent inhibition of fibrinolysis: role of carboxypeptidase-U and the premature lysis of clots from hemophilic plasma. *Blood* 1996; 88: 3815–3823.

30 Bajzar L, Manuel R, Nesheim ME. Purification and characterization of TAFI, a thrombin-activable fibrinolysis inhibitor. *J Biol Chem* 1995; 270: 14477–14484.

31 De Kruyff B, Van Dijck PW, Demel RA, Schuijff A, Brants F, Van Deenen LL. Non-random distribution of cholesterol in phosphatidylcholine bilayers. *Biochim Biophys Acta* 1974; 356: 1–7.

32 Zwaal RF, Roelofsen B, Comfurius P, van Deenen LL. Organization of phospholipids in human red cell membranes as detected by the action of various purified phospholipases. *Biochim Biophys Acta* 1975; 406: 83–96.

33 Renooij W, Van Golde LM, Zwaal RF, Van Deenen LL. Topological asymmetry of phospholipid metabolism in rat erythrocyte membranes. Evidence for flip-flop of lecithin. *Eur J Biochem* 1976; 61: 53–58.

34 Zwaal RF, Comfurius P, van Deenen LL. Membrane asymmetry and blood coagulation. *Nature* 1977; 268: 358–360.

35 Chap HJ, Zwaal RF, van Deenen LL. Action of highly purified phospholipases on blood platelets. Evidence for an asymmetric distribution of phospholipids in the surface membrane. *Biochim Biophys Acta* 1977; 467: 146–164.

36 Bevers EM, Comfurius P, van Rijn JL, Hemker HC, Zwaal RF. Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. *Eur J Biochem* 1982; 122: 429–436.

37 Bevers EM, Comfurius P, Dekkers DW, Zwaal RF. Lipid translocation across the plasma membrane of mammalian cells. *Biochim Biophys Acta* 1999; 1439: 317–330.

38 Sims PJ, Wiedmer T, Esmon CT, Weiss HJ, Shattil S. Assembly of the platelet prothrombinase complex is linked to vesiculation of platelet plasma membrane. Studies in Scott syndrome: An isolated defect in platelet procoagulant activity. *J Biol Chem* 1989; 264: 17049–17054.

39 Dachary-Prigent J, Toti F, Satta N, Pasquet JM, Uzan A, Freyssinet JM. Physiopathological significance of catalytic

Cambridge Books Online © Cambridge University Press, 2009

phospholipids in the generation of thrombin. *Semin Thromb Hemost* 1996; 22: 157–164.
40 Dachary-Prigent J, Freyssinet JM, Pasquet JM, Carron JC, Nurden AT. Annexin V as a probe of aminophospholipid exposure and platelet membrane vesiculation: a flow cytometry study showing a role for free sulfhydryl groups. *Blood* 1993; 81: 2554–2565.
41 Wolf BB, Goldstein JC, Stennicke HR et al. Calpain functions in a caspase-independent manner to promote apoptosis-like events during platelet activation. *Blood* 1999; 94: 1683–1692.
42 Siljander P, Carpen O, Lassila R. Platelet-derived microparticles associate with fibrin during thrombosis. *Blood* 1996; 87: 4651–4663.
43 Nomura S, Shouzu A, Nishikawa M, Kokawa T, Yasunaga K. Significance of platelet-derived microparticles in uremia. *Nephron* 1993; 63: 485.
44 Nomura S, Yasunaga K. Influence of platelet-derived microparticles on coagulation in a lung cancer patient receiving chemotherapy. *Chest* 1993; 103: 979–980.
45 Lundahl TH, Lindahl TL, Fagerberg IH, Egberg N, Bunescu A, Larsson A. Activated platelets and impaired platelet function in intensive care patients analyzed by flow cytometry. *Blood Coagul Fibrinol* 1996; 7: 218–220.
46 Hugel B, Socie G, Vu T, Toti F, Gluckman E, Freyssinet JM, Scrobohaci ML. Elevated levels of circulating procoagulant microparticles in patients with paroxysmal nocturnal hemoglobinuria and aplastic anemia. *Blood* 1999; 93: 3451–3456.
47 Nomura S, Kagawa H, Ozaki Y, Nagahama M, Yoshimura C, Fukuhara S. Relationship between platelet activation and cytokines in systemic inflammatory response syndrome patients with hematological malignancies. *Thromb Res* 1999; 95: 205–213.
48 Omoto S, Nomura S, Shouzu A et al. Significance of platelet-derived microparticles and activated platelets in diabetic nephropathy. *Nephron* 1999; 81: 271–277.
49 Nieuwland R, Berckmans RJ, McGregor S, Boing AN, Romijn F P, Westendorp RG, Hack CE, Sturk A. Cellular origin and procoagulant properties of microparticles in meningococcal sepsis. *Blood* 2000; 95: 930–935.
50 Byzova TV, Plow EF. Networking in the hemostatic system: integrin alphaIIbbeta3 binds prothrombin and influences its activation. *J Biol Chem* 1997; 272: 27183–27188.
51 Scandura JM, Ahmad SS, Walsh PN. A binding site expressed on the surface of activated human platelets is shared by factor X and prothrombin. *Biochemistry* 1996; 35: 8890–8902.
52 Nesheim ME, Furmaniak-Kazmierczak E, Henin C, Cote G. On the existence of platelet receptors for factor V(a) and factor VIII(a). *Thromb Haemost* 1993; 70: 80–86.
53 Altieri DC. Xa receptor EPR-1. *Faseb J* 1995; 9: 860–865.
54 Ahmad SS, Scandura JM, Walsh PN. Structural and functional characterization of platelet receptor-mediated factor VIII binding. *J Biol Chem* 2000; 275: 13071–13081.
55 Loscalzo J, Inbal A, Handin RI. von Willebrand protein facilitates platelet incorporation in polymerizing fibrin. *J Clin Invest* 1986; 78: 1112–1119.
56 Ahmad SS, Rawala-Sheikh R, Walsh PN. Comparative interactions of factor IX and factor IXa with human platelets. *J Biol Chem* 1989; 264: 3244–3251.
57 Ahmad SS, Rawala-Sheikh R, Cheung WF, Jameson BA, Stafford DW, Walsh PN. High-affinity, specific factor IXa binding to platelets is mediated in part by residues 3–11. *Biochemistry* 1994; 33: 12048–12055.
58 Hoffman M, Monroe DM, Roberts HR. Coagulation factor IXa binding to activated platelets and platelet- derived microparticles: a flow cytometric study. *Thromb Haemost* 1992; 68: 74–78.
59 Ho DH, Baglia FA, Walsh PN. Factor XI binding to activated platelets is mediated by residues R(250), K(255), F(260), and Q(263) within the apple 3 domain. *Biochemistry* 2000; 39: 316–323.
60 Greengard JS, Heeb MJ, Ersdal E, Walsh PN, Griffin JH. Binding of coagulation factor XI to washed human platelets. *Biochemistry* 1986; 25: 3884–3890.
61 Baglia FA, Walsh PN. Prothrombin is a cofactor for the binding of factor XI to the platelet surface and for platelet-mediated factor XI activation by thrombin. *Biochemistry* 1998; 37: 2271–2781.
62 Oliver JA, Monroe DM, Roberts HR, Hoffman M. Thrombin activates factor XI on activated platelets in the absence of factor XII. *Arterioscler Thromb Vasc Biol* 1999; 19: 170–177.
63 Osterud B, Rao LV, Olsen JO. Induction of tissue factor expression in whole blood: lack of evidence for the presence of tissue factor expression in granulocytes [In Process Citation]. *Thromb Haemost* 2000; 83: 861–867.
64 Dormann D, Clemetson KJ, Kehrel BE. The GPIb thrombin-binding site is essential for thrombin-induced platelet procoagulant activity [In Process Citation]. *Blood* 2000; 96: 2469–2478.
65 Reverter JC, Beguin S, Kessels H, Kumar R, Hemker HC, Coller BS. Inhibition of platelet-mediated, tissue factor-induced thrombin generation by the mouse/human chimeric 7E3 antibody. Potential implications for the effect of c7E3 Fab treatment on acute thrombosis and 'clinical restenosis'. *J Clin Invest* 1996; 98: 863–874.
66 Herault JP, Lale A, Savi P, Pflieger AM, Herbert JM. In vitro inhibition of heparin-induced platelet aggregation in plasma from patients with HIT by SR 121566, a newly developed Gp IIb/IIIa antagonist [letter]. *Blood Coagul Fibrinol* 1997; 8: 206–207.
67 Herault JP, Peyrou V, Savi P, Bernat A, Herbert JM. Effect of SR121566A, a potent GP IIb-IIIa antagonist on platelet- mediated thrombin generation in vitro and in vivo. *Thromb Haemost* 1998; 79: 383–388.
68 Keularts IM, Beguin S, de Zwaan C, Hemker HC. Treatment with a GPIIb/IIIa antagonist inhibits thrombin generation in platelet rich plasma from patients. *Thromb Haemost* 1998; 80: 370–1.
69 Kumar R, Beguin S, Hemker HC. The influence of fibrinogen and fibrin on thrombin generation – evidence for feedback activation of the clotting system by clot bound thrombin. *Thromb Haemost* 1994; 72: 713–721.

Cambridge Books Online © Cambridge University Press, 2009

70 Béguin S, Kumar R. Thrombin, fibrin and platelets, a resonance loop in which von Willebrand factor is a necessary link. *Thromb Haemost* 1997; 78: 590–594.
71 Béguin S, Kumar R, Keularts I, Seligsohn U, Coller BS, Hemker HC. Fibrin-induced platelet procoagulant activity in clotting plasma, role of GPIIb-IIIa and GPIb and von Willebrand factor. 1999;
72 Béguin S, Kumar R, Keularts I, Seligsohn U, Coller BS, Hemker HC. Fibrin-dependent platelet procoagulant activity requires GPIb receptors and von Willebrand factor. *Blood* 1999; 93: 564–570.
73 Keularts IM, Hamulyak K, Hemker HC, Beguin S. The effect of DDAVP infusion on thrombin generation in platelet-rich plasma of von Willebrand type 1 and in mild haemophilia A patients [In Process Citation]. *Thromb Haemost* 2000; 84: 638–642.

Cambridge Books Online © Cambridge University Press, 2009

27

Platelets and chemotaxis

Stefania Momi and Paolo Gresele

Division of Internal and Cardiovascular Medicine, Department of Internal Medicine, University of Perugia, Italy

Introduction

Platelets have been considered mostly for their role in hemostasis, thrombosis and vascular disease; however, many studies over the last few decades have provided an emerging body of evidence on their potential role in host defence reactions. This unconventional function of platelets is consistent with the theory that platelets may represent the phylogenetic vestige of the primitive hemocytes of lower organisms[1]. As hemocytes, which exert a central role both in defence against invading microorganisms and in repair of tissue injury, platelets maintain in many regards the function of inflammatory cells, able to activate complement, secrete proteases, alter vascular permeability and tone and express cytotoxic properties. For instance, platelets have been shown to participate in cytotoxicity against the parasites responsible for shistosomiasis, toxoplasmosis, and trypanosomiasis[2–6].

Although platelets are anucleated cells, they have an anatomical structure and biochemical properties in many aspects similar to leukocytes and it is thus conceivable that platelets may participate in the defence against infections and in other inflammatory responses. For instance, several observations have confirmed the involvement of platelets in allergic disease and in particular in the tissue inflammatory changes associated with allergic asthma[7]. However, given the role of paramount importance that platelets play in hemostasis and thrombosis and considering the complicacy of the mechanisms regulating these functions, which have absorbed much of the research efforts on these cells, little attention has been paid to the mechanisms regulating platelet inflammatory activities. This chapter will discuss functional activities of platelets related to chemotaxis, an important step in cell involvement in tissue inflammation.

Chemotaxis in eukaryotic cells

Cell migration plays a central role in a wide variety of biological phenomena, both physiologic and pathologic, going from embryogenesis to tumour metastatization[8].

In the inflammatory response, circulating leukocytes migrate by diapedesis across the wall of microvessels into the damaged area where they act as inflammatory cells displaying phagocytic and immune functions. The molecular components involved in cell migration are now largely identified and it is possible to make some generalizations across a wide spectrum of migrating cell types, including amoebae, leukocytes, fibroblasts, neurons and platelets, looking for similarities among physical mechanisms and biochemical characteristics[8].

The process can be sequenced in rolling on the endothelial monolayer, tight adhesion and transmigration, steps involving the active participation of both the transmigrating cells and the endothelium.

Migration of circulating cells begins with the capture or tethering of the cells from the flowing blood and their rolling along the vessel wall where, after appropriate stimuli, they become firmly adhered to the endothelium. Firm adhesion is the preliminary step necessary for cell migration into tissues and it involves the interaction with a number of adhesion molecules located on the migrating cell on one side and on the endothelium on the other.

In general, the adhesion processess leading to inflammation are mediated by different groups of adhesion molecules: the selectins, the integrin family, and the intercellular adhesion molecules.

Selectins mediate the process in which neutrophils roll or slow down prior to their adherence to endothelial cells[9]. Three classes of selectins have been identified: L-selectin, which has been identified on leukocytes; E-selectin, on the surface of activated endothelial cells; and P-selectin, found

on endothelial cells and platelets. Selectins function by binding to carbohydrate ligands present on the cognate cell. L-selectin is involved not only in the tethering and rolling of leukocytes on endothelium but also plays an important role in the activation of leukocytes: recently L-selectin has been established as a receptor capable of signal transduction[10].

P-selectin and E-selectin mediate PMN to endothelial cell adhesion. These selectins are expressed on endothelium only when appropriate inflammatory stimuli (e.g. complement products, oxygen-derived free radicals, cytokines) are generated. Moreover, binding of neutrophils to E-selectin can upregulate the adhesive activity of some integrins, particularly Mac-1[9]. P-selectin (GMP140, CD62P) is stored in Weibel–Palade bodies of endothelial cells and in α-granules of platelets[11]. Upon platelet, or endothelium, activation P-selectin is mobilized to the cell surface where it can interact with its ligand on PMNs, the P-selectin glycoprotein ligand-1 (PSGL-1, CD162). Monocytes and platelets also possess PSGL-1 and can thus bind to P-selectin present on activated endothelium. PSGL-1 consists of a disulfide-bound homodimer and is capable of binding two P-selectin ligands simultaneously[12].

The P-selectin to PSGL-1 interaction is short lived and reversible, unless additional adhesive events are soon evoked[13]. Following specific stimuli, P-selectin binding is followed by a fast redistribution of PSGL-1 to uropods on activated PMNs and may signal the transition from rolling to capture[14].

The second part of the adhesion process is tight binding mediated by integrins. Integrins are receptor molecules composed of two non-covalently associated heterodimers, α and β subunits, and are divided into different subfamilies according to β subunit expression[15].

α and β subunits together form an extracellular ligand binding site while the cytoplasmic tails of integrins provide phosphorylation sites and linkages to cytoskeletal proteins involved in signal transduction[16,17].

There are 8 different β-subunits (β1–β8) that associate with one of 16 α-subunits to form at least 23 known receptors in a variety of cells including lymphocytes, polymorphonuclear leukocytes and platelets[18]. Integrins mediate cell–cell binding and cell interactions with extracellular proteins such as laminin, fibronectin, vitronectin and fibrinogen.

The β1 very late antigen (VLA) integrins comprise a family of receptors that mediate cell adhesion to extracellular matrix (ECM) proteins such as collagen, fibronectin and laminin. They share a common β chain (β1, CD29) that is non-covalently linked to one of at least six different α-chains (α1–α6, CD49a–f) determining the binding properties of the receptor[19]. The VLA proteins are specialized transmembrane receptors allowing the attachment of cells to collagen (VLA-2), fibronectin (VLA-4 and –5) and laminin (VLA-6). Platelets, like megakaryocytes, contain VLA-5, VLA-2 and VLA-6[20] while VLA-4 is expressed on all leukocytes[21].

Recent observations indicate that extravasation of PMNs may be associated with up-regulation of β1 integrins on their cell surface[22]. Surface expression of the $\alpha2\beta1$ (VLA-2) integrin is induced in human and rat PMN on extravasation in vivo suggesting that PMNs locomotion and recruitment to extravascular tissue are critically dependent on $\alpha2\beta1$ integrin function[23].

The binding of PMNs to endothelial cells is mediated mainly by macrophage antigen-1 (Mac-1, CD11b/Cd18, $\alpha_M\beta_2$) and lymphocyte-associated function antigen-1 (LFA-1, $\alpha_L\beta_2$, CD11a/CD18). LFA-1 is the prevailing integrin involved in lymphocyte emigration[24]. In addition to mediating specific adherence, Mac-1 specifically participates in chemotaxis, in phagocytosis and in the production of reactive oxygen species. The ligand for Mac-1 is a cell surface protein found on endothelial cells, namely intercellular cell adhesion molecule-1 (ICAM-1)[25].

Integrins appear to exist in either an active or inactive state and the contact with activated endothelium may induce the appearance of activation epitopes on integrins thus favouring adhesion[17].

ICAM-1, ICAM-2 and VCAM-1 are the most important members of the intercellular adhesion molecules that are cell surface proteins involved in antigen recognition, complement binding or cellular firm adhesion[26]. They are characterized by a variable number of extracellular Ig-like domains with conserved cysteine sequences that form disulfide bonds to stabilize β sheets of the tertiary structure[27]. The Ig family of adhesive receptors bind a number of specific counterreceptors expressed on cells involved in the adhesive interactions. LFA-1 is the counterreceptor of ICAM-1 and ICAM-2 and is also expressed on activated platelets[28] while the physiological counterreceptor of VCAM-1 is VLA-4[21]. ICAM-1 and ICAM-2 bind also to Mac-1[29,30].

Once adhered to the endothelium, cells must undergo anatomical changes that allow them to move across the vessel wall. To this end, they must acquire a spatial asymmetry that allows them to translocate as a result of intracellular generated forces[8].

The locomotion of cells through tissue follows the sequential steps of morphological polarization, membrane extension, formation of cell-substratum attachment, contractile force, traction and release of attachment.

These events are mediated by cytoskeletal changes[31]. It is

Cambridge Books Online © Cambridge University Press, 2009

the polymerization and breakdown of F-actin that leads to the formation and retraction of lamellipodia which function like arms and legs of the migrating cell.

In addition, the actin cytoskeleton is a key mediator of cell polarization and of the directed migration of cells towards a chemoattractant. The two most prominent actin filament-containing structures found in migrating cells are stress fibres and lamellipodia.

Stress fibres are bundles of actin filaments associated with myosin II filaments and other proteins forming contractile fibres. They form sites of adhesion at the plasma membrane, called focal contacts or focal adhesions, where transmembrane integrins are clustered and associated with extracellular matrix proteins outside the cell and with a large number of proteins inside the cell[32]. Formation of such focal adhesions and reorganization of the actin cytoskeleton have been shown to be associated with the phosphorylation of components such as focal adhesion kinase (FAK), the adaptor protein p130 Cas, Crk and paxillin that are directly associated with cell migration[33,34].

Lamellipodia are broad, highly dynamic membrane protrusions that extend and retract through a combination of actin polymerization at the plasma membrane, depolymerization within the cytoplasm, and myosin-mediated rearward movements of the actin fibres[35].

Many signalling events have been identified as being involved in cell migration. Rho-like small GTPases, with their main representatives (Rho, Rac and Cdc42), have been recognized as key modulators of the signalling pathways that mediate the cytoskeletal changes required for both cellular motility and cell–cell adhesion[36]. An important action of Rho, Rac and Cdc42 is their ability to regulate the phosphorylation status of myosin light chain, a major regulator of the actin–myosin interaction[37].

Leukocyte-, but also fibroblast- and other cell type-, migration is largely controlled by soluble stimuli defined chemokines. Chemokines (chemotactic cytokines) are a small group of proteins with four conserved cysteines forming two essential disulfide bonds. CXC and CC chemokines are distinguished according to the position of the first two cysteines, which are either adjacent (CC or β chemokines) or separated by one amino acid (CXC or α chemokines)[38]. Recently, lymphotactin has been described, a chemokine with only two conserved cysteines (C), as well as chemokines with three amino acids between the first two cysteines (CX_3C motif)[38]. Most chemokines are produced under pathological conditions by tissue cells and infiltrating leukocytes. Stimulation of leukocyte suspensions with chemokines leads to a fast shape change that involves polymerization of actin filaments, formation of lamellipodia and activation of the integrins that mediate the adhesion to endothelial cells. Moreover, the activation of leukocytes by cytokines induces some other responses such as the rise in intracellular free calcium concentration, the production of oxygen radicals and bioactive lipids, the release of the content of cytoplasmic storage granules, such as proteases from neutrophils and monocytes, histamine from basophils[39], or cytotoxic proteins from eosinophils[40]. The effects of chemokines are mediated by seven transmembrane domain receptors coupled to GTP-binding proteins. Chemokines have two main sites of interaction with their receptors, one in the N-terminal region and the other within an exposed loop of the backbone that extends between the second and the third cysteine[41]. The N-terminal binding site is essential for the triggering of the receptor.

RANTES (regulated on activation, normal T-cell expressed and secreted) is the first chemokine identified as a potent eosinophil attractant and is released upon appropriate stimuli mainly by platelets but also by fibroblasts[42]. RANTES (ligand for CCR1, CCR3 and CCR9 receptors) has been identified in the bronchoalveolar lavage fluid (BAL) of asthmatics exposed to allergen challenge[43]. The observation that RANTES activates eosinophils and basophils, inducing chemotaxis and the release of histamine and leukotrienes, was the first proof that chemokines are involved in allergy[44].

Monocyte chemotactic proteins (MCPs) have been described in the past few years; they include MCP-1, MCP-2, MCP-3, MCP-4 and MCP-5, all ligands for the CCR2 receptor. MCP-3 acts as chemoattractant for eosinophils and can activate basophils[45].

Recently three peptides with eosinophilic chemotactic activity have been identified including eotaxin (CCL11), eotaxin-2 (CCL24) and eotaxin-3 (CCL26). Eotaxin is expressed in the lungs of animals with allergic asthma and in human tissues where eosinophils accumulate. The in vitro effects of eotaxin on eosinophils include chemotaxis, transendothelial migration, induction of the release of reactive oxygen species, induction of Ca^{2+} mobilization and actin polymerization, and induction of CD11b upregulation. In vivo, eotaxin cooperates with IL-5 to induce eosinophil recruitment and mast cell growth[46]. The specific receptor for eotaxin is CCR3 and it is present on eosinophils (40 000–400 000 receptors per cell), basophils, T-lymphocytes with T_{H2} helper properties[47] and platelets[48]. This receptor (CCR3) appears to mediate most of the actions of the CC chemokines, such as RANTES, MCP-2, MCP-3, MCP-4, and MIP-1α, on eosinophils[49].

Other chemokines involved in cell migration include macrophage inflammatory protein (MIP)-1α (ligand for

Cambridge Books Online © Cambridge University Press, 2009

CCR1, CCR5 and CCR6 receptors), MIP-1β (ligand for CCR5 and CCR8), macrophage-derived chemokine (MDC) (ligand for CCR4), thymus- and activation-regulated chemokine (TARC), stromal derived factor-1α (SDF-1α) (ligand for CXCR4) and some interleukins (like Il-8).

Human MIP-1α is a weak eosinophil attractant and has the ability to stimulate histamine release from basophils and mast cells while murine MIP-1α is a potent eosinophil attractant. MIP-1α is enhanced in bronchoalveolar lavage fluid of asthmatics[50].

MDC is a chemoattractant for monocytes, eosinophils and T_{H2} lymphocytes. In a murine model of lung inflammation, antibodies blocking MDC expression lead to a significant reduction of eosinophils in lung[51]. TARC is selectively expressed on the T_{H2} subset of lymphocytes and is of particular relevance for the recruitment and activation of eosinophils[52]. MDC and TARC are chromosome 16q13 chemokines[53] and are both ligands for CCR4.

A chemokine with potential involvement in lymphocyte maturation and other homeostatic functions is SDF-1α that attracts in vivo and in vitro resting lymphocytes with high affinity[54]. It is chemotactic for cells that express CXCR4 receptors, such as T lymphocytes, neutrophils and monocytes[55]; platelets too express the CXCR4 receptor[48]. Recently, a role for SDF-1α, and CXCR4 in transendothelial/stem cell migration and engraftment has been postulated[56].

Chemokines may be released by some cells involved in allergic reactions upon IgEs interaction with their receptor. The high affinity IgE receptor FcεRI (receptor for the Fc region of the ε-chain of IgE) is responsible for immediate hypersensitivity reactions: when a multivalent allergen associates with two or more FcεRI receptor-bound IgE molecules on the cell surface it cross-links receptors inducing degranulation of the cells and rapid release of stored mediators, such as histamine, and synthesis and secretion of cytokines that attract and activate inflammatory cells[57]. FcεRI is expressed on mast cells, basophils, eosinophils, platelets, monocytes and Langerhans cells[58–61]. The low affinity IgE receptor, FcεRII (CD23), is implicated in long-lasting IgE sensitivity. FcεRII is expressed on the surface of various inflammatory cells, such as eosinophils, lymphocytes, macrophages and platelets[62]. IgEs themselves close a negative feedback loop by switching off their own synthesis, through the interaction with FcεRII[57].

In addition to chemokines, a number of cytokines may also contribute to regulate chemotaxis. IL-1 is an important mediator of inflammation and fever[63]. IL-1 induces P-selectin and ICAM-1 expression on endothelial cells and shedding of L-selectin and expression of integrins on PMNs. Although IL-1 is not itself chemotactic for PMNs, its ability to activate endothelial cells causes the expression of selectins and integrins facilitating leukocyte transendothelial migration in vitro[18].

The actual consensus is that leukocyte transmigration requires mechanisms that open endothelial cell junctions to allow leukocyte passage, although another mechanism, transendothelial passage, has also been described in specific models[64]. The PMN to endothelial cell interaction, in the presence of a chemoattractant, induces a rise of intracellular calcium in endothelial cells[65] that results in the creation of gap junctions between adjacent cells through which neutrophils are able to pass[66]. Phosphorylation of myosin light chain kinase (MLCK) is a Ca^{2+}/calmodulin-dependent event and can regulate agonist and flow-stimulated Ca^{2+} influx in endothelial cells[67]. MLCK catalyzes myosin II filament formation and myosin-actin interactions and induces actin polymerization[68]. It was shown that actin filaments bind directly to the adherence junctions-associated protein, α-catenin[69], and that the tight junctions-associated protein ZO-1 binds directly to α-spectrin, a cross-linking protein of the actin filament[70]. On the other hand, actin polymerization can directly open these junctions[70]. Leukocytes may regulate MLCK activity in endothelial cells thus promoting their own transmigration. Some authors had earlier postulated that platelets too could pass through opened interendothelial cell junctions[71].

Recently, it was shown that transmigrating leukocytes cause a focal and reversible disruption of the vascular endothelial-specific cadherin (VE cadherin) complex[72].

Inflammatory cells transmigrate then through the basement membrane towards the site of inflammation using degrading proteinases. Matrix metalloproteinases (MMPs) comprise a family of zinc binding enzymes that degrade proteins of the extracellular matrix[73]. They are secreted by various inflammatory cells as proenzymes that are then activated by partial proteolytic cleavage. Lymphocytes use matrix metalloproteinase 9 (MMP-9 or gelatinase B) and MMP-2 (gelatinase A) to migrate through reconstituted basement membrane[74], neutrophils use MMP-9 and elastase[75] while eosinophils migrate by releasing and activating MMP-9 when activated by both PAF and IL-5[76]. Recently, it was shown that purified mature polyploid megakaryocytes produce and secrete MMP-9 and that MMP-9 is required for in vitro megakaryocyte migration through the components of the basement membrane in response to a chemoattractant stimulus, such as SDF-1α[77]. The enzymatic activity of matrix metalloproteinases is also necessary for megakaryocyte fragmentation and platelet release[77]. MMP-2 and MMP-9 have been reported to be

Cambridge Books Online © Cambridge University Press, 2009

Table 27.1. Chemokines and platelets

Receptor or platelet	Chemokine ligand	Presence of the ligand in platelets (granules)	Responding cells
CCR1	MIP-1α, RANTES	Yes	Eosinophils, monocytes, activated T cells, dendritic cells, platelets
CCR3	Eotaxin, RANTES	Yes	Eosinophils, basophils, TH2 cells, platelets
CCR4	TARC, MDC	No	Activated T cells, dendritic cells, Th2 cells, platelets
CXCR4	SDF-1α	No	Dendritic cells, monocytes, resting T cells, platelets
Unknown	HRF	Yes	Basophils, mast cells
Unknown	βTG	Yes	Lymphocytes, monocytes, neutrophils, fibroblasts
Unknown	PF4	Yes	Lymphocytes, monocytes, neutrophils, fibroblasts, platelets (?)

secreted by collagen-stimulated platelets and active MMP-2 has been shown to play a role in platelet aggregation acting as a pro-aggregatory stimulus[78–80].

Structural characteristics of platelets that imply the ability to migrate

Platelets possess an anatomic and biochemical machinery under many aspects comparable to leukocytes and a series of structural characteristics that may be relevant to inflammation. Moreover, platelets are known to inherit several characteristics from their bone marrow progenitor cells, megakaryocytes, and these are provided with vivacious cell locomotion[81,82] (see Chapter 2).

Concerning the first step in cellular transmigration, i.e. adhesion to the endothelial monolayer, platelets possess and/or express a number of adhesive proteins or adhesive protein counterreceptors. Platelets contain P-selectin, stored in α-granules[83], and express its ligand PSGL-1 on their surface; although platelets can express P-selectin on their surface upon activation, this does not appear to regulate platelet rolling in vivo on activated endothelium[84] while it is probably involved in the crosstalk between platelets and leukocytes[85]. On the other hand, platelets interact with both P- and E-selectin exposed on activated endothelium[84,86] through their constitutively expressed PSGL-1 receptor[87]. Platelets have been observed while rolling in vivo on an activated endothelial surface in a manner which appears to be very similar to that observed for leukocytes: both cell types roll on stimulated vessel wall and for both this process is dependent on the expression of endothelial P-selectin[84].

The role of different platelet integrins in the adhesion process is a widely investigated and characterized process (see Chapters 5, 21 and 42).

Concerning the intracellular changes required to express cell locomotion, platelets have a cytoskeletal framework that allows cell motion (see Chapter 6).

In resting platelets the discoid shape is maintained by a network of actin filaments, spectrin and integrins, that together form the membrane skeleton, a submembranous structure that coats internally the cytoplasmic surface of platelets[88], and by actin gel filaments that link this structure to transmembrane proteins[89,90]. After stimulation, the activation of low molecular weight G proteins, such as Rac-1, induces the formation of focal complexes, very dynamic structures that, as the cells spread, are replaced by focal adhesions. Additional cytoskeletal changes lead to the formation of stress fibres, under the induction of RhoA[91], a member of the Rho family of low molecular weight GTPases. Stress fibres associate with focal adhesions allowing a contractile response to be exerted on the extracellular integrin-associated ligands. The continuous formation of filopodia and lamellipodia that leads to focal complexes is due to actin dynamic polymerization[92]. One of the signalling molecules that is activated as a consequence of integrin-induced signals is calpain. Platelets contain in their cytosol µ-calpain (one of the two major forms) that becomes activated when platelets aggregate in response to stimuli or when they spread on extracellular matrix proteins[89]. Calpain, by inducing Rac-1 and RhoA activation, provokes integrin-induced formation of focal adhesions and actin filament reorganization[89].

Concerning the soluble stimuli that control migration, recent reports have demonstrated the surface expression of different chemokine receptors on platelets (Table 27.1). The receptors for chemokines are seven transmembrane domain structures linked to G-proteins and mediate calcium flux upon activation[93,94]. Recently it was shown, by using flow cytometry, immunoprecipitation, Western blotting and reversible transcriptase polymerase chain reaction, that human platelets express CCR1, CCR3, CCR4 and CXCR4 chemokine receptors[48,95.] The effect of various

Cambridge Books Online © Cambridge University Press, 2009

cytokines and chemokines (IL-8, MCP-3, MCP-1, MIP-1α, eotaxin, RANTES, Thymus activation-regulated chemokine [TARC], MDC and SDF-1α), acting on different receptors, on Ca^{2+} levels in platelets was also tested. Most of the ligands tested gave clear calcium signals. A cellular response to MIP-1α and RANTES implicates the presence of the CCR1 receptor. A response to eotaxin and RANTES implicates the presence of CCR3, while a response to TARC and MDC implicates the existence of the CCR4. Finally, a response to SDF-1α implicates the expression of the CXCR4 receptor[48]. Although it is not yet clear what is the function of these chemokine receptors on platelets, reports have suggested that some chemokines may be able to activate platelets.

There is earlier evidence that PF4, an α-granule protein of platelets which is also a CXC chemokine[96], may bind to the platelet surface and may modulate platelet aggregation and secretion induced by low levels of platelet agonists[97] even though a specific receptor for PF4 has yet to be identified and no further data confirming this early report have appeared in the literature. Recently Abi-Younes and coworkers demonstrated that SDF-1α, a chemokine highly expressed in human atherosclerotic plaques, induces platelet aggregation and calcium signalling[98].

Most recently, MDC and TARC too, in addition to SDF-1α, were shown to induce platelet activation, in a rapid (less than 5 s) and maximal way under arterial flow conditions, by facilitating the agonistic activity of low doses of primary agonists such as ADP or thrombin. TARC, MDC and SDF-1α increase intracellular calcium concentration in the presence of ADP and their effects are insensitive to indomethacin, thus independent of cyclooxygenase[99]. The effects of MDC and TARC on platelets are mediated by their common receptor CCR4[100].

Challenge with SDF-1α or MDC also exposes P-selectin on the platelet surface[99].

Platelets themselves contain some of the chemokines that are ligands for CCR1 and CCR3, such as RANTES or MIP-1α[101,102], thus one role of the chemokines may be that of feeding back to receptors on the same or other platelets to amplify the response to stimuli. On the other hand, platelets do not contain either CCR4 (MDC, TARC) or CXCR4 (SDF-1α) agonists. Therefore, the role of these receptors may be to involve platelets in situations where these agonists are provided by other cells[48] and it can be hypothesized that they may be involved in platelet migration.

Activated platelets have been shown to exhibit membrane-bound IL-1 bioactivity. Using immunocytological and flow cytometric techniques IL-1α and IL-β were found in the cytoplasm of both resting and thrombin activated-platelets[103]; IL-1 is able to influence indirectly the transendothelial migration of leukocytes[18]. Moreover, flow cytometry studies have shown that the surface of platelets is able to express IL-1R and IL-8R (receptors for Il-1 and IL-8, respectively), the expression of which is significantly increased in inflammatory bowel disease[104].

It has already been mentioned that IgEs may induce the release of chemokines from inflammatory cells in allergic conditions[105,106]. Platelets of atopic individuals release RANTES and other chemokines stored in their α-granules in response to IgE[107]. Platelets can be activated by IgEs and are involved in IgE mediated effector mechanisms (see Chapter 54) and in IgE-mediated allergic disorders (see Chapter 56).

Concerning the passage of platelets through endothelium, it is interesting to observe that activated platelets can produce a rise of intracellular Ca^{2+} in endothelial cells[108], a condition favouring MLCK activation and the breakdown of intercellular junctions[68,72], thus allowing passage between endothelial cells. On the other hand, in a model of skin inflammation induced by fMLP injection, transendothelial cell migration[109], which does not require loosening of intercellular junctions, was also observed.

Finally, regarding the extracellular matrix degradation required for inflammatory cells to migrate through the basement membrane, platelets have been shown to contain and release MMPs (MMP-2 and MMP-9) in vitro and in vivo[78,80].

MMP-2 is secreted by platelets upon stimulation with natural agonists, such as collagen or thrombin, and active MMP-2 has been shown to play a role in platelet activation. MMP-2 is secreted as zymogen and is activated on the cell surface by MT-MMPs (membrane type matrix metalloproteinase), anchored at the cell membrane by transmembrane and intracellular domains[78]. Pro MMP-2 activation occurs through the formation of the trimolecular complex MT1MMP/TIMP2/MMP2 and recent studies have shown the existence of MT1–MMP and TIMP-2 (tissue inhibitor of MMP-2) in platelets and their role in aggregation[79]. In vivo release of MMP2 by platelets activated at a site of vessel wall damage in vivo in humans was recently reported[80].

Observations on platelet chemotaxis in vitro

It was only in the early 1970s that studies on platelet migration were reported. Before the publication of these results, platelets were considered simply passive elements in flowing blood and platelet motion in vitro, as observed under light microscopy, appeared to be a manifestation of Brownian movements[110]. Platelet migration in vitro has

Cambridge Books Online © Cambridge University Press, 2009

been studied for the first time by Lowenhaupt in 1972, by using a modification of the method used for studying macrophage migration in delayed hypersensitivity reactions[111,112], and her observations were confirmed by other laboratories in the following years. She used a capillary tube, closed by flame at one end and filled with platelet rich plasma. Each tube was centrifuged to obtain a platelet pellet, the capillaries were then broken 1–1.5 mm above the platelet pellet level and they were secured in a Petri-dish (migrating chamber), 20 mm in diameter and 3 mm deep, carefully filled with buffer medium and covered with a siliconized and sterilized cover glass. The chambers were then placed in a CO_2 incubator at 22 °C for 1 to 18 hours. After incubation the specimens were observed with a stereomicroscope and recorded photographically[113]. That platelet migration was not merely passive diffusion or Brownian motion but rather an active, energy requiring, process was shown by the demonstration that metabolic inhibitors, such as iodoacetamide acid, sodium fluoride and 2,4-dinitrophenol, inhibited platelet movement[114]. The study of cell migration in vitro was greatly facilitated by the introduction of the chamber of Boyden[115], a method initially developed for the study of leukocyte chemotaxis, and still largely employed to this end, which was adapted to the study of platelet migration by Valone and coworkers[116]. The method consists of the use of a perspex chamber, constituted by two compartments separated by an 8 μm micropore filter, 100 μm thick. A platelet suspension is added to the upper compartment while the lower compartment contains buffer medium. During the incubation period platelets migrate inside the filter. After 3 hours of incubation the filters are removed, washed, fixed in 10% formalin, stained and mounted on a glass slide. Migration into each filter is quantitated by counting the number of platelets at a fixed level (in the studies of Valone, 40–70 μM) from the surface of the filter where the platelet suspension was laying. Using this method it was shown that platelets not only migrate in a random fashion but also migrate specifically and directionally toward chemotactic stimuli such as collagen and PGE1[116]. Platelets are extremely susceptible to aggregation and thus the conditions for collection and handling were selected to minimize such a possibility.

Later, Lowenhaupt and coworkers developed a new method to study platelet migration by using gel-filtered platelets resuspended in autologous platelet free plasma radiolabelled with ^{111}In-oxine and were induced to migrate in a specially designed 7-compartment chamber[117]. This method allowed a more quantitative assay of platelet migration and thus a more precise comparison of the chemotactic properties of various substances, including soluble substances. With this method she demonstrated that collagen induces chemotaxis without the need for direct contact with platelets, since migration was observed when a filter was interposed between platelets and the stimulus. She postulated the existence of 'chemotaxins' produced by the interaction between collagen and plasma able to pass through the filter and to stimulate the migration of platelets toward collagen[118]. They also showed that different sources and forms of collagen have different chemotactic effects on platelets: rat-tail tendon collagen was a better inducer of platelet migration than bovine tendon collagen. Moreover, they showed that the structural features of collagen required for inducing aggregation are distinct from those required for inducing chemotaxis; fibrillar formation, needed to induce aggregation, was not necessary to induce chemotaxis[117].

Factors which were shown to influence blood platelet migration are: temperature, pH, anticoagulant, incubation time and buffer composition. Nathan[118] reported that platelet migration was maximal at 30 °C while Duquesnoy[119] observed a higher migration at 25 °C. Lowenhaupt observed little or no migration at 4 °C while, in her working conditions, migration was optimal at temperatures ranging from 22 to 37 °C[114]. On the basis of these data Lowenhaupt carried out her subsequent experiments at 22 °C. Valone observed maximal platelet migration between 30 to 37 °C and used 37 °C as the standard temperature for examining all the other variables in subsequent experiments[116].

In a pH-range curve of platelet migration, maximal migration was observed by Valone between pH 6 and 6.5 with then a plateau between 6.5 and 8.5[116]. Lowenhaupt noticed that no or little migration occurred at pH 6 while migration increased markedly from pH 6 to 7.4.

The effect of anticoagulants on platelet migration was studied by Duquesnoy and Lowenhaupt. Duquesnoy showed that the inhibitory effect of antiplatelet antibodies on migration was more evident when platelets were derived from citrated blood and based on this observation he chose to use sodium citrate for his experiments[119]. Lowenhaupt showed that migration was optimal in preparations containing sodium citrate or ACD while with other anticoagulants, such as EGTA, Na–EDTA, Mg–Na–EDTA and sodium oxalate, the migration rate was reduced and heparin consistently inhibited migration[114].

The effect of incubation time was tested by Valone, Nathan and Duquesnoy[116,118,119]. Valone showed that when platelets are incubated from 1 to 5 hours, maximal migration is observed at the longest incubation time[116]. For Nathan platelets migrate rapidly for the first 12 hours and then reach a plateau[118]. Duquesnoy observed a quick

Cambridge Books Online © Cambridge University Press, 2009

migration in platelets obtained from blood anticoagulated with EDTA, showing complete migration in the first 30 min while platelets obtained from citrated blood complete their migration in 12 hours[119].

Finally, the influence of the composition of the buffer used for platelet suspension on platelets' ability to migrate was assessed by Valone[116]. He found that platelet migration was optimal in the absence of added magnesium and calcium; the latter, when added at concentrations greater than 10^{-4}M, inhibited migration. The medium used by Valone contains thus only glucose and gelatin at concentrations (0.2 g/100 ml and 0.5 g/100 ml, respectively), inferred by dose-response curves, that favour maximal migration[116].

The ability of platelets to migrate in vitro is dependent on the contractile proteins of the cytoskeleton. Indeed, the incubation of platelets with cytochalasin B, which reversibily disrupts cellular actin microfilaments and reversibily stops cell movements, inhibits migration while the incubation with colchicine, which intereferes with the polymerization of microtubules, has no effect; therefore microtubules are not involved in the migration process[120].

While all the above studies have characterized the migration of platelets from healthy donors and the response to physiologic stimuli (collagen, prostaglandins)[117,121], no studies have explored platelet migration in disease and/or in response to pathological stimuli.

The studies carried out by Duquesnoy[119] were aimed at the detection of the platelet perturbing activity of antihuman leukocyte antigen (HLA) and antiplatelet antibodies[119] and used a test of inhibition of the migration of platelets from a capillary tube. This method, a modification of the migration test of Lowenhaupt[113], was as sensitive as the platelet lysis method and more sensitive than complement fixation, aggregometry and platelet factor-3 release for the detection of antiplatelet antibodies[119,122].

Based on observations suggesting the passage of platelets in the extravascular space in patients with allergic asthma (see below), we have studied the migration response of platelets from allergic asthmatics to in vitro stimulation with the specific sensitizing allergen by using the Boyden chamber system described by Valone[116]. Our data show that platelets from asthmatic subjects migrate in response to the specific allergen to which the patients are sensitized[123], but not in response to other allergens, and that IgEs play a role in this phenomenon. Monoclonal antihuman IgE antibodies, but not their Fab fragments, induced the migration of platelets from allergic asthmatics but not from healthy controls. Antihuman IgG antibodies were ineffective. The proposed mechanism is the following: in asthmatic subjects, IgEs produced following previous contacts with the allergen and bound to the platelet Fc IgE receptors, bind the allergen or the specific anti IgE MoAb. The binding process induces the cross-linking of contiguous receptors that activates the cell (personal unpublished observations) with a process already described for mast cells and basophils[124] (Fig. 27.1). Actually, the cross-linking of IgE receptors on platelets has been shown to induce other platelet activities, such as cytotoxicity against parasites[125], oxygen radical formation[126], and release of chemokines[107,127].

Observations on platelet chemotaxis in vivo

Studies in animals that imply the ability of platelets to migrate

Although very few direct studies on in vivo platelet migration exist, several observations imply the ability of platelets to migrate into tissues.

Degranulated platelets have been found extravascularly in the bronchial tissue, in close contact with contracted smooth muscle cells, in animals challenged with an allergen or with platelet activating factor (PAF)[128,129]. Moreover, platelets have been recovered in the broncho-alveolar lavage fluid of rabbits during the late-response after an allergen challenge[130]. The presence of platelets in these specimens does not appear to be the consequence of blood extravasation as no other blood cells were concomitantly found. The presence of platelets outside vessels, accompanied by neutrophils, was described also in rat skin after the intradermal injection of PAF[131].

Radiolabelled platelets localize in lungs after intravenous challenge with some platelet agonists, like ADP or collagen[132], or after challenge with allergen or PAF[132,133]. However, while they rapidly reenter the circulation with most of the agonists used, suggesting embolization and subsequent dissolution of the platelet microthrombi, a long-lasting retention is observed after PAF challenge, which suggests platelet diapedesis into bronchial tissue[134].

PAF- or allergen-challenge induce eosinophil infiltration in lungs of normal and allergic animals. Selective platelet depletion with an antiplatelet antiserum reduced PAF and antigen-induced eosinophil infiltration into the lungs suggesting that platelets are mediators in this phenomenon[135].

Recently, it was shown that injection of lipopolysaccharides (LPS) or endotoxin in mice and rats induced a slow accumulation of platelets in the sinusoidal and perisinusoidal Disse spaces of the liver. Considering that blood platelets of mice contain a large amount of 5HT, the

Cambridge Books Online © Cambridge University Press, 2009

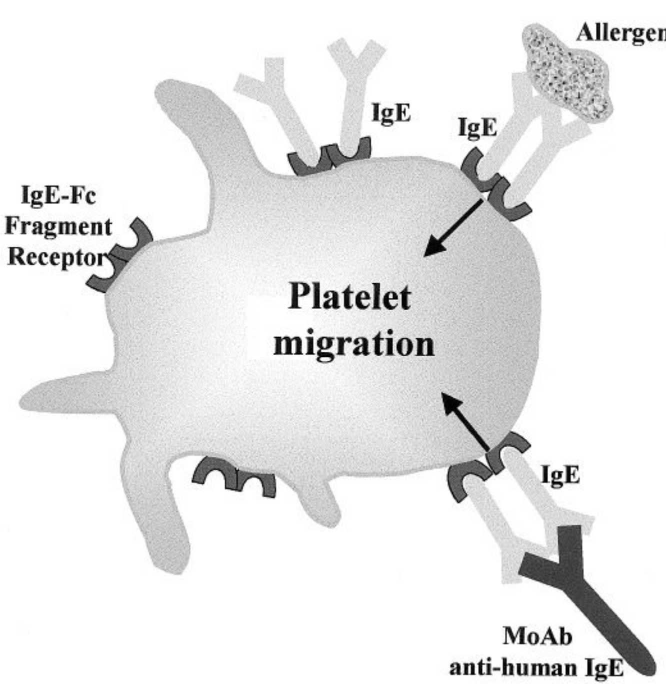


Fig. 27.1. Hypothetical mechanism of the allergen-induced chemotaxis of platelets from patients with allergic asthma. The cross-linking of adjacent, IgE-bound, IgEs receptors transduces a signal leading to chemotaxis. A complete monoclonal antibody antihuman IgE produces the same effect.

authors suggested that a sensitive measure of the diapedesis of platelets into tissues is the measurement of the local levels of 5HT[136]. Most of the platelets accumulated in these extravascular spaces still retained their intact structure. They also showed that the injection of chemokines TNF and IL-1 induce an accumulation of 5HT in the liver. The authors conclude that in response to IL-1, TNF or LPS platelets translocate into the liver in a way which is independent from aggregation and that some of them enter the hepatocytes[136].

Studies in animals showing platelet migration

The appearance of platelets outside blood vessels in acute inflammation is a classic observation[137,138] but little was known until recently about the mechanisms by which platelets extravasate. Recently, an interesting work of Feng et al.[109] has demonstrated active transendothelial migration of platelets by serial electron-microscopy of thin tissue sections from guinea pigs in which acute tissue inflammation was provoked by the skin injection of fMLP[64].

In this study it was shown that platelets in the vascular lumen extend pseudopods towards the luminal endothelial cell plasma membrane to provide their attachment: evidence that platelets participate actively in diapedesis.

In addition, it was observed that single platelets adhering to activated endothelium were completely enclosed within endothelial cytoplasmic vacuoles, generally located towards the periphery of the endothelial cells close to interendothelial cell junctions (Fig. 27.2). Platelets within endothelial cell cytoplasmic vacuoles extended pseudopod-like processes, again showing an active role in the process (Fig. 27.3). Subsequently, platelet-containing vacuoles opened to the endothelial cell ablumen whereupon platelets were discharged into the underlying basal lamina (Fig. 27.4)[105]. Following transmigration across the basal lamina, platelets passed into the dermal connective tissue where they were found free together with neutrophils and other white cells. Despite the close contact with matrix components, migrating platelets did not display the ultrastructural features of a classic release reaction[109].

The mechanism of transmigration observed with this model demonstrates that platelets cross undamaged the endothelium by a process similar to phagocytosis, a phenomenon not dissimilar to that observed for neutrophils with the same model[64]. However, the fact that platelets migrated in the extracellular space were not activated in this model must not necessarily apply to other conditions in which the inflammatory stimulus inducing diapedesis is different, like bacteria, allergens or other noxious agents.

Cambridge Books Online © Cambridge University Press, 2009

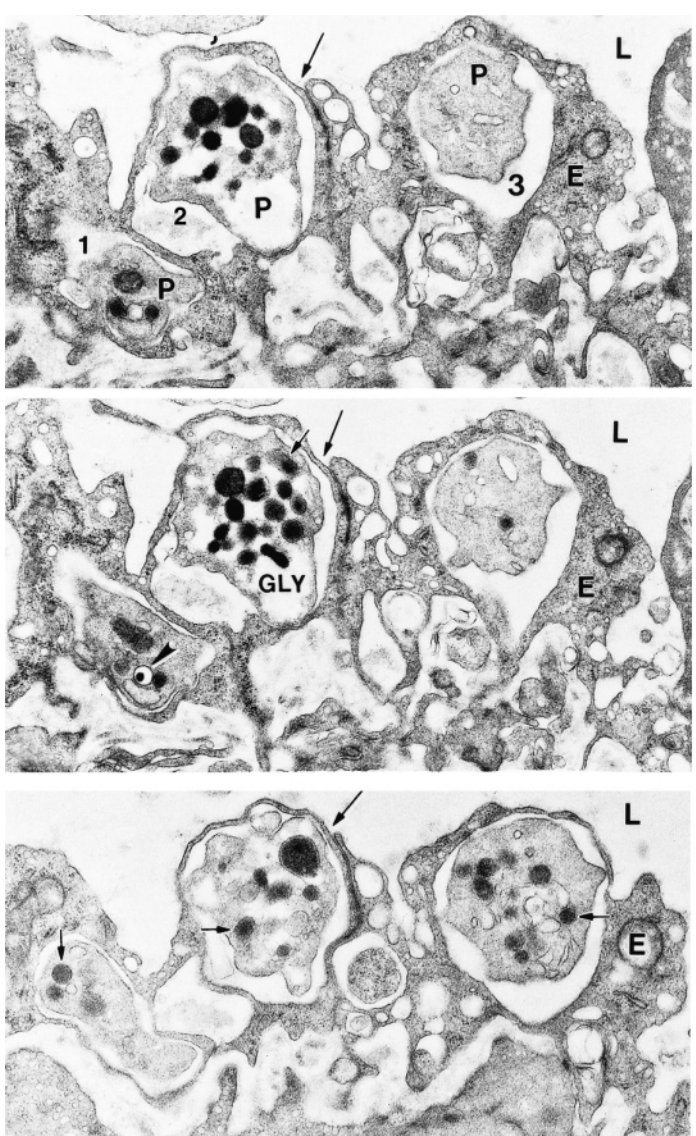


Fig. 27.2. Electron micrographs showing transcellular passage of three platelets across endothelial cells. 1, 2 and 3 are cytoplasmic endothelial vacuoles containing single platelets (P). Each platelet is entirely contained in the vacuole. The endothelium (E) shows no signs of injury. Each platelet presents intact α- and dense-granules. $\times$16 500. (Reproduced from ref. 109 with the permission of Professor Ann M. Dvorak.)

Actually, transendothelial migration too is not necessarily the only mechanism operating in vivo and active migration through interendothelial cell junctions may also take place[71,139].

Studies in humans

Platelet aggregates have been observed in the lamina propria of the microvasculature of lungs of asthmatic subjects during late-onset airway obstruction following allergen provocation[140]. Platelets were also found in the bronchoalveolar lavage fluid in asthmatics after allergen bronchoprovocation; some platelets were degranulated and free granules in the lavage were also observed[141]. Furthermore, platelets have been actually observed undergoing diapedesis in the lungs of asthmatics. Bioptic specimens taken from bronchi of subjects with asthma showed platelets passing through gap junctions between endothelial cells of postcapillary venules[139]. Moreover, platelet diapedesis has been observed towards the epithelial bronchial surfaces of symptomatic atopic asthmatics. Bronchial biopsy from these subjects showed platelets and electron-dense fibrils resembling fibrin, together with membranous debris, at the luminal edge from where surface epithelium was lost. All these observations suggest that, like leukocytes, platelets migrate into inflamed tissue. Platelets were often found near macrophages in bronchoalveolar lavage also after local antigen challenge into a subsegmental airway[130].

Another interesting observation concerns the early stages of the formation of atherosclerotic lesions during which monocytes, attached to inflamed endothelial cells and entering the subendothelial space by diapedesis, have been observed by immuno histochemical staining; interestingly, platelets adhering to the membrane of migrating monocytes were evident[142].

It may not come as a surprise that histologic observations on platelet migration in vivo are so scanty in the literature as the small dimensions of these cells and, especially, the lack of a nucleus which stains clearly in histologic specimens, render their detection rather difficult. Careful electron microscopic observation and/or special stainings are required and it is likely that, when applied on a larger scale, this will lead to a much more frequent detection of platelets in inflamed tissues.

Cambridge Books Online © Cambridge University Press, 2009

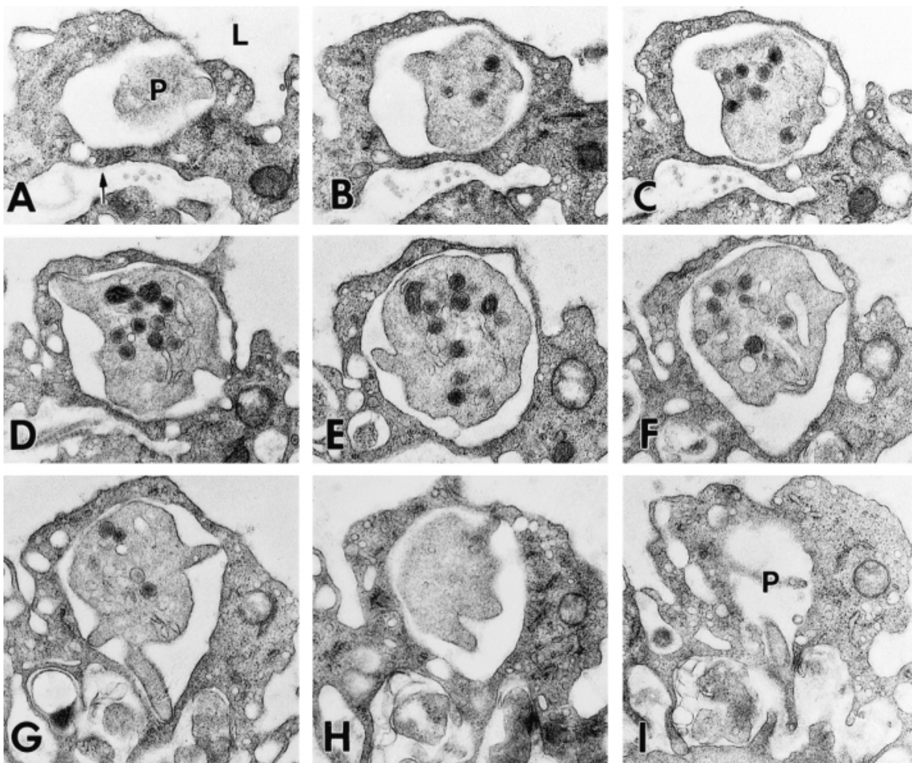


Fig. 27.3. Electron micrographs showing the active migration through the endothelium of one platelet contained in a endothelial cell vacuole. The platelet (P) in (*a*) is included in a cytoplasmic vacuole of the endothelial cell that is near the lumen (L). From (*b*) to (*g*) it is possible to see distinctly the granules inside the platelet. Extension of platelet pseudopods is evident. The centre of the cell recedes leaving a granule-free area (*h*) and a single process (P) visible in (*i*) × 16 500 (reproduced from ref. 109 with the permission of Professor Ann M. Dvorak).

Substances in platelets relevant to the chemotaxis of other cells

Besides their own ability to migrate, platelets may exert an important influence on the diapedesis of other cells. Upon activation platelets release several mediators, either stored in their granules or cytoplasm or synthesized after stimulation, that may participate in eliciting and maintaining the inflammatory reaction. Among these substances, a number of mediators of chemotaxis are present; moreover, platelets may directly, by cell contact, influence the migrating properties of other cells (Table 27.2).

Platelet δ granules store, among other substances, serotonin which is vasoactive, vasopermeabilizing and stimulating for fibroblasts and it is known to act as an inflammatory mediator[143]. In addition, serotonin enhances the chemotactic responsiveness of human monocytes and the chemotaxis of polymorphonuclear leukocytes[144]. Serotonin-stimulated human blood mononuclear cells secrete lymphocyte chemoattractant activity (IL-16) that may promote, via serotonin type 2 receptors, the recruitment of CD4+ T lymphocytes into an inflammatory focus[145].

Platelet α granules contain several proteins including PGDF, TGF-β, PF4, βTG, RANTES, MIP-1α, NAP-2 precursors and P-selectin, which may play a role in cell migration.

Platelets have also been shown to contain another chemokine of the CXC class: epithelial neutrophil-activating protein 78 (ENA-78)[95], which induces migration in neutrophils expressing the CXCR2 (IL-8RB) receptor on their surface[146].

PGDF and TGFβ are growth factors which display vasoactive and chemotactic properties on smooth muscle cells, macrophages, monocytes and fibroblasts[147,148]. Recently, PDGF and TNFβ released by aggregating platelets have been shown to act synergistically to induce the

Cambridge Books Online © Cambridge University Press, 2009

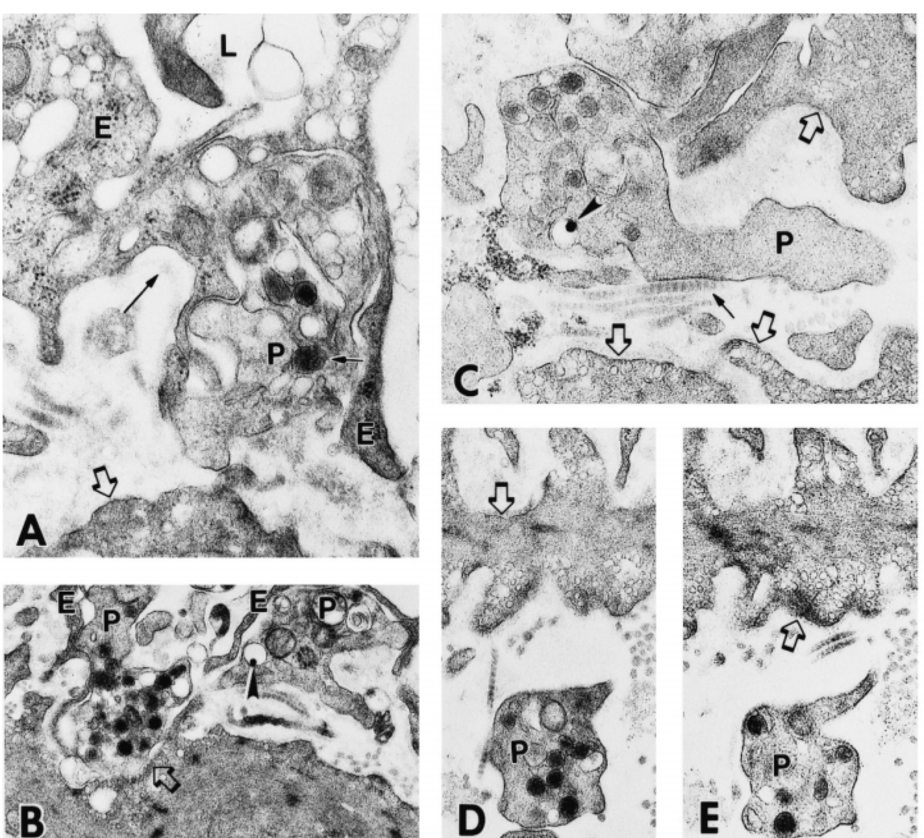


Fig. 27.4. Electron micrograph showing the later stages of platelets' transendothelial migration. (*a*) The platelet (P) has already crossed the overlying endothelial (E) cell and basal lamina (black arrow) and has come into contact with an underlying pericyte (open arrow). α-granules (small arrow) are evident in the platelet. (*b*) One platelet (P) beneath the endothelium is partially surrounded by thin endothelial cell processes and has indented an underlying pericyte (open arrow). A second platelet resides beneath the endothelial cell process and contains a granule with a dense core, characteristic of serotonin-containing platelet granules. The dense granule is indicated. (*c*) This platelet (P) also contains a single dense core granule and multiple α-granules. It lies beneath the endothelium close to pericyte processes (open arrows). Note that the elongated platelet process of this normally granulated platelet extends along a strand of interstitial collagen (closed arrow). (*d*), (*e*) Serial sections showing a platelet (P) that, after emerging through the endothelium, has migrated beneath the pericyte above it (open arrow). The platelet exhibits minor shape change and cytoplasmic extensions but granules are unaltered. (*a*) $\times$ 31 000; (*b*) $\times$ 14 000; (*c*) $\times$ 22 000; (*d*) $\times$ 18 000; (*e*) $\times$ 18 000. (Reproduced from ref. 109 with the permission of Professor Ann M. Dvorak.)

expression on smooth muscle cells of vascular endothelial growth factor (VEGF)[149], an endothelial mitogen and chemoattractant that is also able to induce endothelial cell migration[150].

PF4 is the major platelet α-chemokine. Purified PF4 lacks chemotactic activity for polymorphonuclear cells but in the presence of TNFα it stimulates these cells to undergo such functions as exocytosis of secondary granule markers or tight adhesion to different surfaces[151]. PF4 not only affects PMN, but it also mediates the release of histamine by basophils[152] and plays a role in eosinophil adhesion[153]. Beta-thromboglobulin (βTG) released from platelet α-granules exerts a chemotactic activity for neutrophils, monocytes, lymphocytes and fibroblasts[154,155].

RANTES and MIP-1α, 8 kDa peptides belonging to the CC chemokines, are potent chemotactic factors for basophils and eosinophils. RANTES is chemotactic also for memory T lymphocytes and for monocytes[42,157]. MIP-1α is less powerful in attracting basophils and eosinophils but is much more potent in inducing a transient intracellular Ca^{2+} elevation[158]. MIP-1α has also histamine-releasing activity on basophils and displays chemotactic properties for CD8 T lymphocytes[159].

RANTES is released from α granules of platelets after

Cambridge Books Online © Cambridge University Press, 2009

Table 27.2. Substances in platelets that influence the migration of other cells

Substances released by platelets	Migratory responding cells
Serotonin	Monocyte, PMN leukocytes
PDGF	Endothelial cells
TNFβ	Endothelial cells
PF4	PMN leukocytes, basophils, eosinophils
RANTES	Basophils, eosinophils, memory T cells lymphocytes, monocytes
MIP-1α	Basophils, eosinophils, CD8 + lymphocytes
βTG Ag	PMN leukocytes
IL-8	PMN leukocytes
IL-1	Endothelial cells, smooth muscle cells
Histamine	PMN leukocytes, endothelial cells
Lysosomal enzymes	Fibroblasts, neutrophils
PAF	PMN leukocytes
PGE_2	Monocytes
TXA_2	Endothelial cells
PECAM-1	Neutrophils
CD40L	Endothelial cells

stimulation with thrombin[156]. MIP-1α is observed within cisterns of the open canalicular system and on the plasma membrane before its release from activated platelets[101].

RANTES has been found to be increased in the blood of patients with asthma during spontaneous attacks in a way that correlates with βTG rises suggesting a platelet source of the RANTES detected[160].

Platelet P-selectin, stored in α-granules and mobilized to the plasma membrane upon activation, mediates rolling and adhesion of leukocytes. In vitro neutrophils have been shown to roll on P-selectin of immobilized activated platelets[161].

Platelet λ-granules, the lysosomes, contain enzymes such as cathepsin, β-hexosoaminidase and heparinase, that are released in vitro upon strong stimulation[162,163]. Recently, platelet lysosome release has been demonstrated in vivo in humans at a localized site of vessel wall damage[162]. These acid hydrolases may participate in inflammation and cell diapedesis through their cytotoxic and tissue-degrading activity by remodelling the inflamed tissues.

Platelets contain in their cytoplasm other important mediators of inflammation such as histamine, IL-1 and CD40L, that may be released and can induce chemotaxis.

Histamine is released by platelets upon challenge with histamine releasing factors (HRF), upon aggregatory stimuli and by immunological stimuli in atopic subjects. Platelet histamine represents one of the most important pools of histamine in blood, after that of basophils and monocytes[164]. Histamine increases the number of PMNs migrating across the endothelial cells[165] by altering the endothelial cytoskeleton and increasing eicosanoid release[166].

IL-1, a central mediator in the cytokine cascade and a potent activator of vascular cytokine production, is contained in platelets and released upon activation[167–169] and it thus facilitates leukocyte and fibroblast transmigration.

CD40 ligand (CD40L, CD154), a transmembrane protein structurally related to TNFα originally identified in CD 4+ T cells, has now been identified also on the surface of activated platelets[170]. CD40L has been localized in the platelet cytoplasm by immunoprecipitation and Western blotting and it translocates to the cell surface within seconds of activation in vitro and in the process of thrombus formation in vivo[171]. CD40L induces endothelial cells to secrete chemokines and to express adhesion molecules like ICAM-1, VCAM-1 and E-selectin[26], thus modulating the leukocyte to endothelium interaction.

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a member of the Ig gene superfamily that is expressed on the surface of platelets, endothelial cells and certain leukocyte subsets. PECAM-1 may be an important mediator of leukocyte/endothelial cell interactions[172] and plays a role in leukocyte migration through the endothelium and the perivascular basement membrane[173].

In addition to the granule-stored substances, platelets release lipidic mediators upon activation, such as platelet activating factor (PAF) or arachidonate metabolites. PAF is an acetylated phosphoglyceride derived from the phospholipids of cell membranes and it is a potent proinflammatory mediator able to promote adhesive interactions between leukocytes and endothelial cells leading to transendothelial migration of leukocytes[174,175].

Activated platelets release some arachidonate metabolites, including thromboxane A2 (TxA2) or prostaglandin E2 (PGE2), that may also influence the migration of other cells. TxA2, a potent platelet activator and a vasoconstrictor[176], has a stimulatory activity on the proliferation of smooth muscle cells of the vessel wall[177] and the airways and it may stimulate endothelial cell migration[178]. Recently, it was shown that PGE2 inhibits the potential of monocytes to migrate in response to stimuli such as PAF and C5a and to adhere to endothelial cells. This inhibition is most probably due to a modulation of the chemokine receptor CCR5 and of the β2 integrin Mac-1[179].

Cambridge Books Online © Cambridge University Press, 2009

Conclusions

Chemotaxis is a central phenomenon in the participation of cells to tissue inflammation, allowing the recruitment of inflammatory cells to the site of tissue damage and the local release of cytotoxic and vasoactive mediators. Platelets appear to display the ability to migrate and to influence the migration of a number of other cells involved in inflammation, a property regulated by mechanisms which are different from those involved in platelet aggregation. These functional properties of platelets, together with other findings, characterize the platelet as an inflammatory cell, besides its more studied function of haemostatic element.

A more intensive investigation on the mechanisms regulating this 'unconventional' functional activity of platelets as well as studies on platelet chemotaxis in disease may allow a deeper insight in the mechanisms leading to inflammation and allergy and may open new horizons in the search for drugs limiting these pathologic processes.

References

1 Weksler BB. Platelets. In Gallin JI, Goldstein IM, Snyderman R (ed.). *Inflammation: basic principles and clinical correlates.* New York: Raven Press. 1988; 543–557.
2 Joseph M, Auriault C, Capron MA, Vorng H, Viens P. A new function for platelets: IgE-dependent killing of schistosomes. *Nature* 1983; 303: 810–812.
3 Bout D, Joseph M, Pontet M, Vorng H, Deslee D, Capron A. Rat resistance to schistosomiasis: platelet-mediate cytotoxicity induced by C-reactive protein. *Science* 1986; 231: 153–156.
4 Yong EC, Chi EY, Frietsche TR, Henderson WR. Human-platelet-mediated cytotoxicity against *Toxoplasma gondii:* role of thromboxane. *J Exp Med* 1991; 173: 65–78.
5 Umekita LF, Mota I. In vitro lysis of sensitized *Trypanosoma cruzi* by platelets: role of C3b receptors. *Parasite Immunol* 1989; 11: 561–566.
6 Momi S, Perito S, Mezzasoma AM, Bistoni F, Gresele P. Involvement of platelet in experimental mouse trypanosomiasis: evidence of mouse platelet cytotoxicity against *Trypanosoma equiperdum. Exp Parasitol* 2000; 95: 136–143.
7 Gresele P. The platelet in asthma. In Page CP (ed). *The platelet in health and disease.* Oxford: Blackwell Scientific Publications. 1991; 132–157.
8 Lauffensburger DA, Horwitz AF. Cell migration, a physically integrated molecular process. *Cell* 1990; 81: 359–369.
9 Bevilacqua MP, Nelson RM. Selectins. *J Clin Invest* 1993; 91: 379–387.
10 Hwang ST, Singer M, Giblin PA, Yednock TA, Bacon KB, Simon SI, Rosen SD. GlyCAM-1, a physiologic ligand for L-selectin, activates beta 2 integrins on naive peripheral lymphocytes. J Exp Med 1996; 184: 1343–1348.
11 Malik AR, Lo SK. Vascular endothelial adhesion molecules and tissue inflammation. *Pharmacol Rev* 1996; 48: 213–229.
12 McEver RP, Cummings RD. Role of PSGL-1 binding to selectins in leukocyte recruitment. *J Clin Invest* 1997; 100: 485–492.
13 Davenpeck KL, Steeber DA, Tedder TF, Buchner BS. P- and L-selectin mediate distinct but overlapping functions in endotoxin-induced leukocyte-endothelial interactions in rat mesenteric microcirculation. *J Immunol* 1997; 159: 1977–1986.
14 Bruehl RE, Moore KL, Lorant DE et al. Leukocyte activation induces surface redistribution of P-selectin glycoprotein ligand-1. *J Leukoc Biol* 1997; 61: 489–499.
15 Springer TA. Adhesion receptors of the immune system. *Nature* 1990; 346: 425–434.
16 Alpin HE, Howe S, Alahari SK, Juliano RL. Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin–cell adhesion molecules, and selectins. *Pharmacol Rev* 1998; 50: 197–203.
17 Gerli R, Paolucci C, Gresele P et al. Salicylates inhibit adhesion and transmigration of T lymphocytes by preventing integrin activation induced by contact with endothelial cells. *Blood* 1998; 92: 2389–2398.
18 Wagner JG, Roth RA. Neutrophil migration mechanisms, with an emphasis on the pulmonary vasculature. *Pharm Rev* 2000; 52: 349–374.
19 Hemler ME. VLA proteins in the integrin family. Structures, functions, and their role on leukocytes. *Annu Rev Immunol* 1990; 8: 365–400.
20 Molla A, Mossuz P, Berthier R. Extracellular matrix receptors and the differentiation of human megakaryocytes in vitro. *Leuk Lymphoma* 1999; 33: 15–23.
21 Tiisala S, Hakkaraianen M, Majuri ML, Mattila P, Renkonen R. Down-regulation of monocytic VLA-4 leads to a decreased adhesion to VCAM-1. *FEBS Lett* 1993; 332: 19–23.
22 Werr J, Xie X, Hedqvist P, Rouslahti E, Lindbom L. $\beta 1$ integrins are critically involved in neutrophil locomotion in extravascular tissue in vivo. *J Exp Med* 1998; 187: 2091–2096.
23 Werr J, Johansson J, Eriksson EE, Hedqvist P, Rouslahti E, Lindbom L. Integrin $\alpha 2\beta 1$ (VLA-2) is the principal receptor used by neutrophils for locomotion in extravascular tissue. *Blood* 2000; 95: 1804–1809.
24 Li X, Abdi K, Racun J, MacKey CR, Mentzer SJ. LFA-1 and L-selectin regulation of recirculating lymphocyte tethering and rolling on lung endothelium. *Am J Respir Cell Mol Biol* 1996; 14: 398–406.
25 Van de Stolpe A, Van deer Saag PT. Intercellular adhesion molecule-1. *J Mol Med* 1996; 74: 13–33.
26 Carlos TM, Harlan JM. Leukocyte-endothelial adhesion molecules. *Blood* 1994; 84: 2068–2101.
27 Williams AF, Barclay AN. The immunoglobulin superfamily. *Ann Rev Immunol* 1988; 6: 381–405.
28 McCaffery PJ, Berridge MV. Expression of the leukocyte functional molecule (LFA-1) on mouse platelets. *Blood* 1986; 67: 1757–1764.

Cambridge Books Online © Cambridge University Press, 2009

29 Foy DS, Ley K.Intercellular adhesion molecule-1 is required for chemoattractant-induced leukocyte adhesion in resting, but not inflamed, venules in vivo. *Microvasc Res* 2000; 60: 249–60.
30 Nakashima Y, Sun DH, Trindade MC et al. Signaling pathways for tumor necrosis factor-alpha and interleukin-6 expression in human macrophages exposed to titanium-alloy particulate debris in vitro. *J Bone Joint Surg Am* 1999; 81: 603–15.
31 Mitchison TJ, Cramer LP. Actin-based cell motility and cell locomotion. *Cell* 1996; 84: 371–379.
32 Burtridge K, Chazanowska Wodnika M. Focal adhesion, contractility, and signaling. *Annu Rev Cell Biol* 1996; 12: 463–518.
33 Cowin P, Burke B. Cytoskeleton–membrane intreractions. *Curr Opin Cell Biol* 1996; 8: 56–65.
34 Wang JF, Park IW, Groopman JE. Stromal cell-derived factor-1a stimulates tyrosine phosphorylation of multiple focal adhesion proteins and induces migration of hemopoietic progenitor cells: roles of phosphoinositide-3 kinase and protein kinase C. *Blood* 2000; 95: 2505–2513.
35 Sheetz MP, Falsenfeld D, Galbraith CG, Choquet D. Cell migration as a five step cycle. *Biochem Soc Symp* 1999; 65: 233–243.
36 van Nieuw Amerongen, van Hinsbergh VW. Cytoskeletal effects of rho-small guanine nucleotide-binding proteins in the vascular system. *Arterioscler Thromb Vasc Biol* 2001; 21: 300–311.
37 Evers EE, Zondag GC, Malliri A et al. Rho family proteins in cell adhesion and cell migration. *Eur J Cancer* 2000; 36: 1269–1274.
38 Teran LM. CCL chemokines and asthma. *Immunol Today* 2000; 21: 235–241.
39 Proudfoot AE, Gatto R, Wells TN, Bacon KB. Isolation of a lipid-soluble histamine releasing factor from human platelets. *Biochem Biophys Res Commun* 1994; 201: 443–448.
40 Baggiolini M, Dewald B, Moser B. Human chemokines: an update. *Annu Rev Immunol* 1997; 15: 675–705.
41 Clark-Lewis I, Kim KS, Rajarathnam K et al. Structure–activity relationship of chemokines. *J Leukoc Biol* 1995; 57: 703–711.
42 Kameyoshi Y, Dorschner A, Mallet AI, Christophers E, Schroder JM. Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. *J Exp Med* 1992; 176: 587–592.
43 Teran LM. Eosinophil recruitment following allergen challenge is associated with the release of the chemokine RANTES into asthmatic airways. *J Immunol* 1996; 157: 1806–1812.
44 Baggiolini M, Dahinden CA. CC chemokines in allergic inflammation. *Immunol Today* 1994; 15: 127–133.
45 Stafford S, Li H, Forsythe PA, Ryan M, Bravo R, Alam R. Monocyte chemotactic protein-3 (MCP-3)/fibroblast-induced cytokine CFC in eosinophilic inflammation of the airways and the inhibitory effects of an anti-MCP-3/FIC antibody. *J Immunol* 1997; 158: 4953–4960.
46 Palframan RT, Collins PD, Severs NJ, Rothery S, Williams TJ, Rankin SM. Mechanism of acute eosinophils mobilization from the bone marrow stimulated by interleukin-5: the role of specific adhesion molecules and phosphatidylinositol-3-kinase. *J Exp Med* 1998; 188: 1621–1632.
47 Sallusto F, Mackay CR, Lanzavecchia A. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science* 1997; 277: 2005–2007.
48 Clemetson KJ, Clemetson JM, Proudfoot AEI, Power CA, Baggiolini M, Wells TNC. Functional expression of CCR1, CCR3, CCR4 and CXCR4 chemokine receptors on human platelets. *Blood* 2000; 96: 4046–4054.
49 Ponath PD, Qin S, Post TW et al. Molecular cloning and characterization of a human eotaxin receptor expressed selectively on eosinophils. *J Exp Med* 1996; 183: 2437–2448.
50 Holgate ST, Bodey KS, Janezic A, Frew AJ, Kaplan AP, Teran LM. Release of RANTES, MIP-1α and MCD-1 into asthmatic airways following endobronchial allergen challenge. *Am J Respir Crit Care Med* 1997; 156: 1377–1383.
51 Gonzalo JA, Pan Y, Lloyd CM et al. Mouse monocyte-derived chemokine is involved in airway hyperreactivity and lung inflammation. *J Immunol* 1999; 163: 403–411.
52 Kaplan AP. Chemokines, chemokine receptors and allergy. *Int Arch Allergy Immunol* 2001; 124: 423–431.
53 Greaves DR, Häkkinen T, Lucas AD et al. Linked chromosome 16q13 chemokines, macrophage-derived chemokine, fractalkine and thymus- and activation-regulated chemokine, are expressed in human atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 2001; 21: 923–929.
54 Bleul CC, Fuhlbrigge RC, Casanovas JM, Aiuti A, Springer TA. A highly efficacious lymphocyte chemattractant, stromal derived factor-1 (SDF-1α). *J Exp Med* 1996; 184: 1101–1109.
55 Bleul CC, Farzan M, Choe H, Parolin C, Clark-Lewis I, Sodroski J, Springer TA. The lymphocyte chemoattractant SDF-1 is a ligand for LESR/fusin and blocks HIV-1 entry. *Nature* 1996; 382: 829–833.
56 Gazitt Y. Recent developments in the regulation of peripheral blood stem cell mobilization and engraftment by cytokines, chemokines, and adhesion molecules. *J Hematother Cell Res* 2001; 10: 229–236.
57 Sutton BJ, Gould HJ. The human IgE network. *Nature* 1993; 366: 421–428.
58 Capron A, Dessaint JP, Capron M, Pierce R. Schistosomiasis: from effector and regulation mechanisms in rodents to vaccine strategies in humans. *Immunol Invest* 1992; 21: 409–422.
59 Bieber T, de la Salle H, Wollenberg A et al. Human epidermal Langerhans cells express the high affinity receptor for immunoglobulin E (Fc epsilon RI). *J Exp Med* 1992; 175: 1285–1291.
60 Falcone F, Haas H, Gibbs BF. The human basophil: a new appreciation of its role in immune responses. *Blood* 2000; 96: 4028–4038.
61 Hasegawa S, Pawanakar R, Suzuki K et al. Functional expression of the high affinity receptor for IgE (FceRI) in human platelets and its intracellular expression in human megakaryocytes. *Blood* 1999; 93: 2543–2551.
62 Tunon de Lara M. Immunoglobulins E and inflammatory cells. *Rev Mal Respir* 1996; 13: 27–36.

Cambridge Books Online © Cambridge University Press, 2009

63 Le J, Vilcek J. Tumor necrosis factor and interleukin-1: cytokines with multiple overlapping biological activities. *Lab Invest* 1987; 56: 234–248.

64 Feng D, Nagy JA, Pyne K, Dvorak HF, Dvorak AM. Neutrophils emigrate from venules by a transendothelial cell pathway in response to FMLP. *J Exp Med* 1998; 187: 903–915.

65 Huang AJ, Manning JE, Bandak TM, Ratau MC, Hanser KR, Silverstein SC. Endothelial cell cytosolic free calcium regulates neutrophil migration across monolayers of endothelial cells. *J Cell Biol* 1993; 120: 1371–1380.

66 Wen-Hong S, Hsiun-Ing C, Ji-Ping H, Chauying JJ. Endothelial $[Ca^{++}]$i signaling during transmigration of polymorphonuclear leukocytes. *Blood* 2000; 96: 3816–3822.

67 Watanabe H, Takahashi R, Zhang XX et al. An essential role of myosin light chain kinase in the regulation of agonist- and fluid flow-stimulated Ca2+-influx in endothelial cells *FASEB J* 1998; 12: 341–348.

68 Stull JT, Tansey MG, Tang DC, Word RA, Kamm KE. Phosphorylation of myosin light chain kinase: a cellular mechanism for Ca^{2+} desensitization. *Mol Cell Biochem* 1993; 127–128: 229–237.

69 Rimm DL, Koslov ER, Kebriaei P, Cianci CD, Morrow JS. Alpha 1(E)-catenin is an actin-binding and building protein mediating the attachment of F-actin to the membrane adhesion complex. *Proc Natl Acad Sci USA* 1995; 92: 8813–8817.

70 Rabiet MJ, Plantier JL, Rival Y, Geneoux Y, Lampugnani MG, Dejana E. Thrombin-induced increase in endothelial permeability is associated with changes in cell-to-cell junction organization. *Arterioscler Thromb Vasc Biol* 1996; 16: 488–496.

71 Marchesi VT. Mechanisms of cell migration and macromolecule transport across the walls of blood vessels. *Gastroenterology* 1966; 51: 875–892.

72 Saito H, Minamiya Y, Kitamura M et al. Endothelial myosin light chain kinase regulates neutrophil migration across human umbilical vein endothelial cell monolayer. *J Immunol* 1998; 161: 1533–1540.

73 Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. *Curr Opin Cell Biol* 1998; 10: 602–608.

74 Xia M, Leppert D, Hauser SL et al. Stimulus specificity of matrix metalloproteinases dependence of human T cell migration through a model basement membrane. *J Immunol* 1996; 156: 160–167.

75 Delclaux C, Delacourt C, d'Ortho MP, Boyer V, Laufma C, Harf A. Role of gelatinase B and elastase in human polymorphonuclear neutrophil migration across basement membrane. *Am J Respir Cell Mol Biol* 1996; 14: 288–295.

76 Okada S, Kita H, George TJ, Gleich GJ, Leiferman KM. Migration of eosinophils through basement membrane components. In vitro: role of matrix metalloproteinase-9. *Am J Respir Cell Mol Biol* 1997; 17: 519–528.

77 Lane WJ, Dias S, Hattori K et al. Stromal-derived factor 1-induced megakaryocyte migration and platelet production is dependent on matrix metalloproteinases. *Blood* 2000; 96: 4152–4159.

78 Sawicki G, Salas E, Murat J, Miszta-Lane H, Radomski MW. Release of gelatinase A during platelet activation mediates aggregation. *Nature* 1997; 386: 616–619.

79 Kazes I, Elalamy I, Sraer JD, Hatmi M, Nguyen G. Platelet release of trimolecular compex component MT1–MMP/TIMP2/MMP2: involvement in MMP2 activation and platelet aggregation. *Blood* 2000; 96: 3064–3069.

80 Falcinelli E, Ciferri S, Gresele P. Platelet release matrix metalloproteinase 2 (MMP) 2 in vivo in humans at a localized site of platelet activation. *Thromb Haemost*, Suppl July 2001; P326.

81 Tavassoli M, Aoki M. Migration of entire megakaryocytes through the marrow-blood barrier. *Br J Haematol* 1981; 48: 25–29.

82 Hamada T, Mohle R, Hasselgesser J et al. Transendothelial migration of megakaryocytes in response to stromal-cell derived factor 1 (SDF-1) enhanced platelet formation. *J Exp Med* 1998; 188: 539–548.

83 Stenberg PE, McEver RP, Schuman MA, Jacques PV, Baiton DF. A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. *J Cell Biol* 1985; 101: 880–886.

84 Frenette PS, Johnson RC, Hynes RO, Wagner DD. Platelets roll on stimulated endothelium in vivo: an interaction mediated by endothelial P-selectin. *Proc Natl Acad Sci USA* 1995; 92: 7450–7454.

85 Evangelista V, Manarini S, Sideri R et al. Platelet/polymorphonuclear leukocyte interaction: P-selectin triggers protein-tyrosine phosphorylation-dependent CD11b/CD18 adhesion: role of PSGL-1 as a signaling molecule. *Blood* 1999; 93: 876–885.

86 Frenette PS, Moyna C, Hartwell DW, Lowe JB, Hynes RO, Wagner DD. Platelet-endothelial interaction in inflamed mesenteric venules. *Blood* 1998; 91: 1318–1324.

87 Frenette PS, Denis CV, Weiss L et al. P-selectin glycoprotein ligand 1 (PSGL-1) is expressed on platelets and can mediate platelet-endothelial interactions in vivo. *J Exp Med* 2000; 191: 1413–1422.

88 Fox JE, Boyles J, Berndt M, Steffen P, Anderson L. Identification of a membrane skeleton in platelets. *J Cell Biol* 1988; 107: 1525–1538.

89 Fox JE. The platelet cytoskeleton. *Thromb Haemost* 1993; 70: 884–893.

90 Hartwig JH, Barkalow K, Azim A, Italiano J. The elegant platelet: signals controlling actin assembly. *Thromb Haemost* 1999; 82: 392–398.

91 Clark EA, King WG, Brugge JS, Symons M, Hynes RO. Integrin-mediated signals regulated by members of the Rho family of GTPases. *J Cell Biol* 1998; 142: 573–586.

92 Nachmias VT, Kavaler J, Jacubowitz S. Reversible association of myosin with the platelet cytoskeleton. *Nature* 1985; 313: 70–72.

93 Murphy PM. The molecular biology of leukocyte chemoattractant receptors. *Annu Rev Immunol* 1994; 12: 593–633.

94 Arai H, Charo IF. Differential regulation of G-protein-medi-

Cambridge Books Online © Cambridge University Press, 2009

ated signaling by chemokine receptor. *J Biol Chem* 1996; 271: 21814–21819.
95 Power CA, Clemetson JM, Clemetson KJ, Wells TN. Chemokine and chemokine receptors mRNA expression in human platelets. *Cytokine* 1995; 7: 479–482.
96 Brandt E, Petersen F, Ludwig A, Ehlert JE, Bock L, Flad HD. The beta thromboglobulins and platelet factor 4: blood platelet derived CXC chemokines with divergent roles in early neutrophil regulation. *J Leukoc Biol* 2000; 67: 471–478.
97 Capitanio AM, Niewiarowski S, Rucinski B et al. Interaction of platelet factor 4 with human platelets. *Biochim Biophys Acta* 1985; 839: 161–173.
98 Abi-Younes S, Sauty A, Mach F, Sukhova GK, Libby P, Luster AD. The stromal cell derived factor 1 chemokine is a potent platelet agonist highly expressed in atherosclerotic placques. *Circ Res* 2000; 86: 131–138.
99 Gear AL, Suttitanamongkol S, Viisoreanu D, Polanowska-Grabowska RK, Raha S, Camerini D. Adenosine diphosphate strongly potentiates the ability of the chemokines MDC, TARC, and SDF-1α to stimulate platelet function. *Blood* 2001; 97: 937–945.
100 Abi-Younes S, Si-Tahar M, Luster AD. The CC chemokines MDC and TARC induce platelet activation via CCR4. *Thromb Res* 2001; 101: 279–289.
101 Klinger MHF, Wilhelm D, Bubel S, Sticherling M, Schröder JM, Kühnel W. Immunocytochemical localization of the chemokines RANTES and MIP-1α within human platelets and their release during storage. *Int Arch All Immunol* 1995; 107: 541–546.
102 McLean A. Regulation of RANTES. *Lancet* 1994; 343: 189–190.
103 Sedlmayr P, Blaschitz A, Wilders-Truschnig M, Tiran A, Dohr G. Platelets contain interleukin-1 alpha and beta which are detectable on the cell surface after activation. *Scand J Immunol* 1995; 42: 209–214.
104 Schaufelberger HD, Uhr MR, McGuhin C. Platelets in ulcerative colitis and Chron's disease express functional interleukin-1 and interleukin-8 receptors. *Eur J Clin Invest* 1994; 24: 656–663.
105 Oliveira SH, Lukacs NW. Stem cell factor and IgE-stimulated murine mast cells produce chemokines (CCL2, CCL17, CCL22) and express chemokine receptors. *Inflamm Res* 2001; 50: 168–174.
106 Gounni AS, Lamkhioued B, Koussih L, Ra C, Renzi PM, Hamid Q. Human neutrophils express the high-affinity receptor for immunoglobulin E (Fc epsilon RI): role in asthma. *FASEB J* 2001;15: 9940–9949.
107 Klouche M, Klinger MH, Kuhnel W, Wilhelm D. Endocytosis, storage, and release of IgE by human platelets: differences in patients with type I allergy and nonatopic subjects. *J Allergy Clin Immunol* 1997;100: 235–241.
108 Ashmore RC, O'Brien RF, Stelzner TJ et al. Aggregating platelets increase intracellular calcium in endothelial cells through release of adenine nucleotides. *Biochem Biophys Res Commun* 1990; 166: 909–915.
109 Feng D, Nagy JA, Pyne K, Dvorak HF, Dvorak AM. Platelets exit venules by a transcellular pathway at site of F-Met peptide-induced acute inflammation in guinea pigs. *Int Arch Allergy Immunol* 1998; 116: 188–195.
110 Chamot EM, Mason CW. Brownian movement. In *Handbook of chemical microscopy*. New York: J Wiley 1947; Vol 1: 221–222.
111 George M, Vaughan JH. In vitro cell migration as a model for delayed hypersensitivity. *Proc Soc Exp Biol Med* 1962; 111: 514–519.
112 Lowenhaupt RW, Glueck HI. Platelet migration and survival in culture medium. In *Proceedings, III Congress of the International Society for Thrombosis and Haemostasis*, Washington DC, 1972; 222.
113 Lowenhaupt RW, Miller MA, Glueck HI. Platelet migration and chemotaxis demonstrated in vitro. *Thromb Res* 1973; 3: 477–486.
114 Lowenhaupt RW, Glueck HI, Millaer MA, Kline DL. Factors which influence blood platelet migration. *J Lab Clin Med* 1977; 90: 37–45.
115 Boyden SJ. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes. *J Exp Med* 1957; 115: 453.
116 Valone FH, Austen KF, Goetzl EJ. Modulation of the random migration of human platelets. *J Clin Invest* 1974; 54: 1100–1106.
117 Lowenhaupt RW. Human platelet chemotaxis can be induced by low molecular substance(s) derived from the interaction of plasma and collagen. *Progr Clin Biol Res* 1982; 89: 269–280.
118 Nathan P. The migration of human platelets in vitro. *Thromb Diath Haemorrhag* 1973; 30: 173–177.
119 Duquesnoy RJ, Lorentsen DF, Aster RH. Platelet migration inhibition: a new method for detection of platelet antibodies. *Blood* 1975; 45: 741–747.
120 Lowenhaupt RW, Glueck HI. The effect of cytochalasin B on platelet migration. *Fed Proc* 1974; 33: 243.
121 Lowenhaupt RW. Human platelet chemotaxis: requirement for plasma factor(s) and the role of collagen. *Am J Physiol* 1978; 235: H23–28.
122 Levine SJ, Brubaker DB, Detection of platelet antibodies using the platelet migration assay. *Am J Clin Pathol* 1983; 80: 43–48.
123 Zhang ML, Selli S, Baglioni A et al. Platelets from asthmatic patients migrate in vitro in response to allergen stimulation. *Thromb Haemost* 1993; 69: 916.
124 Kagey-Sobotka A, MacGlashan DW, Lichtenstein LM. Role of receptor aggregation in triggering IgE mediated reactions. *Fed Proc* 1982; 41: 12–16.
125 Polack B, Peyron F, Auriault C. Platelet cytotoxicity against parasites. *Nouv Rev Fr Hematol* 1991; 33: 317–322.
126 Vargas L, Patino PJ, Rodriguez MF et al. Increase in granulocyte-macrophage-colony-stimulating factor secretion and the respiratory burst with decreased L-selectin expression in hyper-IgE syndrome patients. *Ann Allergy Asthma Immunol* 1999; 83: 245–251.
127 Hasegawa S, Tashiro N, Matsubara t, Fukurawa S, Ra C. A

Cambridge Books Online © Cambridge University Press, 2009

comparison of Fcepsilon RI-mediated RANTES release from human platelets between allergic patients and healthy individuals. *Int Arch Allergy Immunol* 2001; 125: 42–47.
128 Lellouch-Tubiana A, Lefort J, Pirotzky E, Vargaftig BB, Pfister A. Ultrastructural evidence for extravascular platelet recruitment in the lung upon intravenous injection of platelet-activating factor (PAF-acether) to guinea pigs. *Br J Exp Pathol* 1985; 66: 345–355.
129 Lellouch-Tubiana A, Lefort J, Pfister A, Vargaftig BB. Interactions between granulocytes and platelets with the guinea-pig lung in passive anaphylactic shock. Correlations with PAF-acether-induced lesion. *Int Arch Allergy Appl Immunol* 1987; 83: 198–205.
130 Metzger WJ, Sjoerdsma K, Richerson HB et al. Platelets in broncho-alveolar lavage from asthmatic patients and allergic rabbits with allergen-induced late phase responses. *Agent Action* 1987; Suppl 21: 151–159.
131 Pirotzky E, Page CP, Roubin R, Pfister A, Paul W, Bonnet J, Benveniste J. PAF-acether induced plasma exudation in rat skin is independent of platelets and neutrophils. *Microcirc Endoth Lymph* 1984; 1: 107–122.
132 Page CP, Paul W, Morley J. An in vivo model for studying platelet aggreation and disaggregation. *Thromb Haemost* 1982; 47: 210–213.
133 Page CP, Paul W, Morley J. Platelets and bronchospasm. *Int Arch Allergy Appl Immunol* 1984; 74: 347–350.
134 Robertson DN, Page CP. Effect of platelet agonist on airway reactivity and intrathoracic platelet accumulation. *Br J Pharmacol* 1987; 92: 105–111.
135 Lellouch-Tubiana A, Lefort J, Simon MT, Pfister A, Vargaftig BB. Eosinophil recruitment into guinea pig lungs after PAF-acether and allergen administration. Modulation by prostacyclin, platelet depletion, and selective antagonists. *Am Rev Resp Dis* 1988; 137: 948–954.
136 Nakamura M, Shibazaki M, Nitta Y, Endo Y. Translocation of platelets into Disse spaces and their entry into hepatocytes in response to lipopolysaccharides, interleukin-1 and tumor necrosis factor: the role of Kupff cells. *J Hepatol* 1998; 28: 991–999.
137 Florey H. *General pathology.* Philadelphia, Saunders, 1970.
138 Zweifach B, Grant L, McCluskey R (eds) *The inflammatory process.* New York: Academic Press, 1973; 2.
139 Laitinen L, Laitinen A. Platelets. In Holgate ST, Church MK (eds.). *Allergy* 1993; 8.1–8.8.
140 Beasley R, Roche WR, Roberts JA, Holgate ST. Cellualr events in the bronchi in mild asthma and after bronchial provocation. *Am Rev Respir Dis* 1989; 139: 806–817.
141 Metzger WJ, Hunninghake GW, Richerson HB. Late asthmatic responses: inquiry into mechanisms and significance. *Clin Rev Allergy* 1985; 3: 145–165.
142 Masuda J, Ross R. Atherogenesis during low level hypercholesterolemia in the nonhuman primate. Fatty streak formation. *Arteriosclerosis* 1990; 10: 178–187.
143 Dray A. Inflammatory mediators of pain. *Brit J Anaesthesiol* 1995; 75: 1225–1231.
144 Sandler JA, Gallin JI, Vaughan M. Effect of serotonin, carbamylcholine, and ascorbic acid on leukocyte cyclic GMP and chemotaxis. *J Cell Biol* 1975; 62: 480–484.
145 Laberge S, Cruikshank WW, Beer DJ, Center DM. Secretion of IL-16 (lymphocyte chemoattractant factor) from serotonin-stimulated CD8+ T cells in vitro. *J Immunol* 1996; 156: 310–315.
146 Nilsson G, Mikovits JA, Metcalfe DD, Taub DD. Mast cell migratory response to interleukin-8 is mediated through interaction with chemokine receptor CXCR2/interleukin-8RB. *Blood* 1999; 93: 2791–2797.
147 Krettek A, Ostergren-lunden G, Fager G, Rosmond C, Bondjers G, Lustig F. Expression of PDGF receptors and ligand-induced migration of partially differentiated human monocyte-derived macrophages. Influence of IFN-gamma and TGF-beta. *Atherosclerosis* 2001; 156: 267–275.
148 Zeller PJ, Skalak TC, Ponce AM, Price RJ. In vivo chemotactic properties and spatial expression of PDGF in developing mesenteric microvascular networks. *Am J Physiol Heart Circ Physiol* 2001; 280: H2116-H2125.
149 Kronemann N, Booloumie A, Bassus S, Kirchmaier CM, Busse R, Schimi-Kerth VB. Aggregating human platelets stimulate expression of vascular endothelial growth factor in cultured vascular smooth muscle cells through a synergistic effect of transforming growth factor β1 and platelet-derived growth factor AB. *Circulation* 1999; 100: 855–860.
150 Senger DR, Ledbetter SR, Claffey KP, Papadopoulos-Sergiuo A, Peruzzi CA, Detmar M. Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the $\alpha v\beta 3$ integrin, osteopontin and thrombin. *Am J Pathol* 1996; 149: 293–305.
151 Petersen F, Ludwig A, Flad HD, Brandt E. TNFα renders human neutrophils response to platelet factor 4: comparison of PF4 and IL-8 reveals different activity profiles of the two chemokines. *J Immunol* 1996; 156: 1954–1962.
152 Brindley LL, Sweet JM, Goetzl EJ. Stimulation of histamine release from human basophils by human platelet factor 4. *J Clin Invest* 1983; 72: 1218–1223.
153 Hayashi N, Chihara J, Kobayashi Y et al. Effect of platelet activating factor and platelet factor 4 on eosinophil adhesion. *Int Arch Allergy Immunol* 1994; 104 (Suppl 1): 57–59.
154 Senior RM, Griffin GL, Huang JS, Walz DA, Deuel TF. Chemotactic activity of platelet alpha granule proteins for fibroblasts. *J Cell Biol* 1983; 96: 382–385.
155 Scheuerer B, Erns M, Durrbaum-Landmann I et al. The CXC chemokine platelet factor 4 promotes monocyte survival and induces monocyte differentiation into macrophages. *Blood* 2000; 95: 1158–1166.
156 Kameyoshi Y, Dörschner A, Mallet AI, Christophers E, Schroder JM. Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. *J Exp Med* 1992; 176: 587–592.
157 Kuna P, Reddigari SR, Schall JI, Rucinski D, Virsman MY, Kaplan AP. RANTES, a monocyte and T lymphocyte chemo-

Cambridge Books Online © Cambridge University Press, 2009

tactic cytokine releases histamine from human basophils. *J Immunol* 1992; 149: 636–642.
158 Rot A, Krieger M, Brunner T, Bischoff SC, Schall TJ, Dahinden CA. RANTES and macrophage inflammatory protein 1α induce the migration and activation of normal human eosinophil granulocytes. *J Exp Med* 1992; 176: 1489–1495.
159 Taub DD, Conlon K, Lloyd AR, Oppenheim JJ, Kelvin DJ. Preferential migration of activated CD4+ and CD8+ T cells in response to MIP-1α and MIP-1β. *Science* 1993; 260: 355–358.
160 Chihara J, Yasuba H, Tsuba A et al. Elevation of the plasma level of RANTES during asthma attacks. *J Allergy Clin Immunol* 1997; 100: S52–S55.
161 Yeo EL, Sheppard JI, Feverstein IA. Role of P-selectin and leukocyte activation in polymorphonuclear cell adhesion to surface adherent activated platelets under physiologic shear conditions (an injury vessel wall model). *Blood* 1994; 83: 2498–2507.
162 Ciferri S, Emiliani C, Guglielmini G, Orlacchio A, Nenci GG. Platelets release their lysosomal content in vivo in humans upon activation. *Thromb Haemost* 2000; 83: 157–164.
163 Guglielmini G, De Ioanni M, Ciferri S et al. Intracellular signal transduction mechanisms regulating the release of lysosomes by activated human platelets. *Haematologica* 2000; Suppl 5: 85.
164 Masini E, Di Bello MG, Raspanti S et al. The role of histamine in platelet aggregation by physiological and immunological stimuli. *Inflamm Res* 1998; 47: 211–220.
165 Doukas J, Shepro D, Hechtman HB. Vasoactive amines directly modify endothelial cells to affect polymorphonuclear leukocyte diapedesis in vitro. *Blood* 1987; 69: 1563–1569.
166 Doukas J, Shepro D, Hechtman HB. Vasoactive amines and eicosanoids interactively regulate both polymorphonuclear leukocyte diapedesis and albumin permeability in vitro. *Microvasc Res* 1989; 37: 125–137.
167 Hawrylowicz CM, Howells GL, Feldmann M. Platelet-derived interleukin-1 induces human endothelial adhesion molecule expression and cytokine production. *J Exp Med* 1991; 174: 785–790.
168 Hawrylowicz CM, Santoro SA, Platt FM, Unanue ER. Activated platelets express IL-1 activity. *J Immunol* 1989; 143: 4015–4018.
169 Kaplanski G, Porat R, Aiura K, Erban JK, Gelfand JA, Dinarello CA. Activated platelets induce endothelial secretion of interleukin-8 in vitro via an interleukin-1 mediated event. *Blood* 1993; 81: 2492–2495.
170 Henn V, Slupsky JS, Grafe M et al. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 1998; 351: 591–594.
171 Hermann A, Rauch BH, Braun M, Shror K, Weber AA. Platelet CD40 ligand (CD40L)-subcellular localization, regulation of expression, and inhibition by clopidogrel. *Platelets* 2001; 12: 74–82.
172 Muller WA. The role of PECAM-1(CD31) in leukocyte emigration: studies in vitro and in vivo. *J Leukoc Biol* 1995; 57: 523–528.
173 Thompson RD, Noble KE, Larbi KY et al. Platelet-endothelial cell adhesion molecule-1 (PECAM-1) in leukocyte migration through the perivascular basement membrane. *Blood* 2001; 97: 1854–1860.
174 Peplow PV. Regulation of platelet activating factor (PAF) activity in human disease by phospholipase A2 inhibitors, PAF acetylhydrolases, PAF receptor antagonists and free radical scavengers. *Prostagl Leukot Essent Fatty Acids* 1999; 61: 65–82.
175 Kuipers TW, Hakkert BC, Hart MHL, Roos D. Neutrophil migration across monolayers of cytokine prestimulated endothelial cells: a role for platelet activating factor and IL-8. *J Cell Biol* 1992; 117: 565–572.
176 Gresele P, Deckmyn H, Nenci GG, Vermylen J. Thromboxane synthase inhibitors, thromboxane receptor antagonists and dual blockers in thrombotic diseases. *Trends Pharmacol Sci* 1991; 12: 158–163.
177 Koba S, Pakala R, Watanabe T, Katagiri T, Benedict CR. Synergistic interaction between thromboxane A2 and mildly oxidized low density lipoproteins on vascular smooth muscle cell proliferation. *Prostagl Leukot Essent Fatty Acids* 2000; 63: 329–335.
178 Nie D, Lamberti M, Zacharek A, Li L, Szekeres K, Tang K, Chen Y, Honn KV. Thromboxane A(2) regulation of endothelial cell migration, angiogenesis, and tumor metastasis. *Biochem Biophys Res Commun* 2000; 267: 245–251.
179 Zeidler R, Csanady M, Gires O, Lang S, Schmitt B, Wollenberg B. Tumor cell-derived prostaglandin E2 inhibits monocyte function by interfering with CCR5 and Mac-1. *FASEB J* 2000; 14: 661–668.

Cambridge Books Online © Cambridge University Press, 2009

28

Platelet–leukocyte interactions relevant to vascular damage and thrombosis

Chiara Cerletti, Virgilio Evangelista, Roberto Lorenzet and Giovanni de Gaetano*

Istituto di Ricerche Farmacologiche Mario Negri, 'G. Bizzozero' Blood and Vascular Cell Interactions Laboratory and 'A. Taticchi' Atherosclerosis and Thrombosis Unit, Department of Vascular Medicine and Pharmacology, 66030 Santa Maria Imbaro, Italy

**Università Cattolica del Sacro Cuore, Centro di Ricerca e Formazione ad Alta Tecnologia nelle Scienze Biomediche, 86100 Campobasso, Italy*

Platelet–leukocyte interaction: a tribute to Giulio Bizzozero

The interaction of blood platelets with leukocytes at the site of vascular injury was clearly described by Giulio Bizzozero, more than one century ago[1]:

> The blood platelets which are carried by the blood stream are arrested as soon as they reach the lesion of the arterial wall; at first one observes two to six platelets; very rapidly their number grows into hundreds. Usually, among these, a few white blood corpuscles are also arrested.
>
> Every time when a vascular wall is damaged . . . arrest of white blood corpuscles represents a secondary phenomenon and may, perhaps, be caused by increased adhesive properties of blood platelets whereby these cells react with white blood corpuscles which have been brought into contact with them by blood circulation (Fig. 28.1).

This was probably the first observation of the simultaneous involvement of platelets and leukocytes in hemostasis and thrombus formation.

Since then, the presence of leukocytes in hemostatic platelet plugs and arterial thrombi has been repeatedly observed by microscopy, but the general consensus was that these cells were only playing a passive role in thrombus formation being more likely involved in the subsequent repair process. In the past two decades, a number of studies have been performed looking for interactions between platelets and leukocytes to better understand their possible importance in the physiopathology of thrombosis.

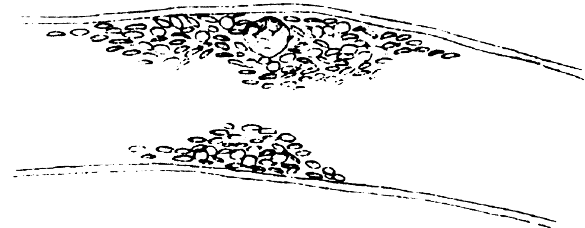


Fig. 28.1. 'Two small mural thrombi which have formed within a small artery of the omentum of a guinea pig. The larger one contains, among blood platelets, a white blood corpuscle.' This figure is reprinted from the original paper of Bizzozero[1], at the occasion of the first centennial anniversary of his death in Turin (April 8, 1901).

Mediators and mechanisms of platelet/leukocyte cross-talk

The broad range of interactions between platelets and leukocytes has been reviewed several times[2–9]. Results from in vitro studies indicate that the mutual modulation of platelet and leukocyte function is extremely complex. Both inhibitory and stimulatory effects have been described, suggesting the possibility of different in vivo physiopathological conditions.

Up-regulation of platelet function by leukocytes

Several leukocyte-derived products, such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), oxygen radical species and the lipid mediator, platelet activating factor (PAF), are endowed with platelet stimulating activity[10–12].

The addition of the leukocyte specific agonist n-formyl-methionyl-leucyl-phenylalanine (fMLP) to human whole blood increases the number of P-selectin-expressing platelets. This increase is inhibited by a PAF antagonist, by the superoxide anion scavenger, superoxide dismutase, and by two different 5-lipoxygenase inhibitors, suggesting the involvement of PAF, superoxide anion and 5-lipoxygenase products in leukocyte-induced platelet activation[13].

Thromboxane (Tx) synthesized by monocytes potentiates ADP-stimulated platelet aggregation of autologous platelets through a fourfold increased binding of ^{125}I-fibrinogen to the platelet surface. Tx generation by monocytes is triggered by the interaction between fibrinogen and its monocyte membrane receptor[14]. Peripheral blood monocytes stimulated with arachidonic acid produce a platelet activating substance derived from cyclo- and lipoxygenase-dependent metabolism[15]; on the other hand, in vitro exposure of monocytes to lipopolysaccharide results in a dose-dependent increase in procoagulant activity and induction of platelet aggregation through thrombin generation[16].

A role for proteinases in PMN-dependent platelet aggregation was indicated by the observation that preincubation of platelets with chymotrypsin-like cationic proteins from human granulocytes resulted in enhanced platelet serotonin release initiated by thrombin or immune complexes[17]. Maria Kopec's group in Poland[18] first provided evidence that a neutral, chymotrypsin-like, serine proteinase purified from human leukocytes, stimulated platelet function. Marked platelet activation was also reported when mixed human platelet–polymorphonuclear (PMN) leukocyte suspensions were challenged with fMLP (or other PMN agonists) in the presence of cytochalasin B, which allows azurophilic granule discharge. Cell-free supernatants of fMLP-activated PMN also induced platelet activation, suggesting a secreted factor was involved[19,20].

Electron microscopic observation of aggregates from mixed cell suspensions showed both cell types closely intermingled: platelets were associated with each other as well as with PMN. Platelets and PMN appeared to interact by very close contact between their membranes (Fig. 28.2). In the aggregates from platelet samples stimulated by PMN-derived supernatant many platelets had lost their granules and were in an advanced stage of shape change, secretion and aggregation[21]. This platelet stimulating activity was identified as cathepsin G[21,22], a neutral serine proteinase contained in the PMN azurophilic granules and released upon activation. Elastase, another serine proteinase stored in the same granules that does not directly stimulate platelets, potentiates platelet activation induced by low concentrations of cathepsin G[23].

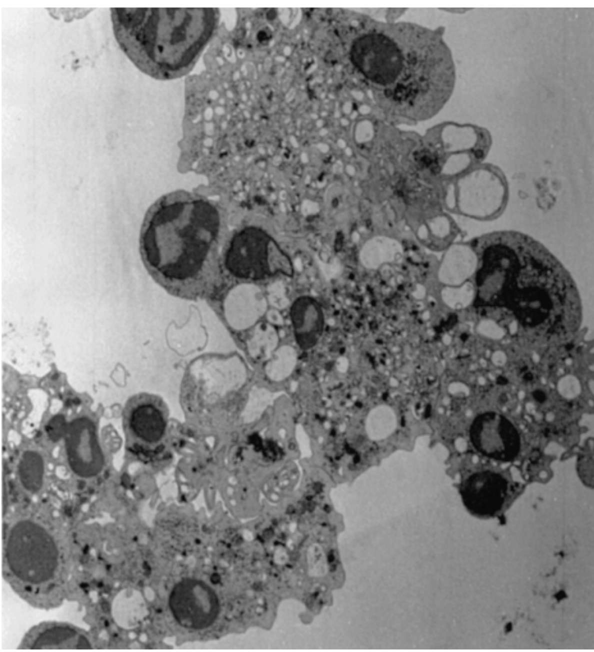


Fig. 28.2. Electron microscopic observation of aggregates from mixed platelet–PMN leukocyte suspensions stimulated with 1 μM fMLP. Both cell types appear closely intermingled: platelets are associated with each other as well as with PMN; platelets and PMN appear to interact by very close contact between their membranes. Original magnification × 9 600. (For more details[21]).

Platelet aggregation and signal transduction mechanisms induced by cathepsin G

Cathepsin G purified from human PMN induces platelet activation at concentrations comparable to those secreted by PMN upon fMLP activation in vitro. Concentration-dependent P-selectin expression[24], serotonin release and Tx formation occur in concomitance with platelet aggregation induced by purified cathepsin G or PMN-activated supernatant[22,25].

Like several other platelet agonists, cathepsin G induces a concentration-dependent (20–500 nM) increase in intraplatelet calcium levels, mainly through an influx across the plasma membrane[22,25]. The calcium increase started by cathepsin G does not depend on cyclooxygenase activation or on exposure of the membrane glycoprotein GpIIbIIIa. Both calcium-dependent and independent mechanisms of protein kinase C activation have been observed in platelets stimulated by cathepsin G[26].

Cathepsin G, thrombin receptors and platelet membrane glycoproteins

Cathepsin G shows saturable reversible binding to human platelets[27]. Since the proteolytic activity is essential for

Cambridge Books Online © Cambridge University Press, 2009

platelet activation by cathepsin G as well as by thrombin, it was suggested that cathepsin G would cause receptor activation by cleavage of the receptor itself. Following the discovery of Protease Activated Receptor 1 (PAR1), the first thrombin receptor cloned[28], attempts were made to determine whether cathepsin G and thrombin work through the same or different receptors. Selak[29] suggested that cathepsin G and thrombin act on different receptors since a monoclonal antibody directed against PAR1 did not block platelets' response to cathepsin G, while inhibiting the response to thrombin. Moreover cathepsin G does not evoke a calcium response in some cell types (human fibroblasts, human umbilical endothelial cells) that respond to thrombin[29,30], nor is cathepsin G able to induce a response in human PAR1-transfected cells[30]. The two enzymes, on the other hand, cross-desensitize[27].

Subsequent work has shown that cathepsin G induces N-terminal PAR1 cleavage at at least two sites: Phe[55]–Trp[56], deleting the tethered ligand domain, and after Arg[41], thus activating the thrombin receptor, when the distal site is altered or otherwise protected[30].

More recently, the platelet activating action of cathepsin G has been shown to be mediated by another protease-activated receptor, indicated as PAR4[31]. Cathepsin G triggered calcium mobilization in washed human platelets, PAR4-transfected fibroblasts and PAR4-expressing *Xenopus* oocytes. An antibody raised against the PAR4 thrombin cleavage site blocked platelet activation by cathepsin G but not by other agonists. Desensitisation with a PAR4 activating peptide had a similar effect. By contrast, inhibition of PAR1 function had no effect on platelet response to purified cathepsin G. PMN-dependent platelet activation was also blocked by the PAR4 antibody. These data show that a specific receptor mediates platelet responses to cathepsin G and increases the biological plausibility of the hypothesis that cathepsin G released from PMN induces platelet activation at sites of vascular injury or inflammation.

In addition to activating platelets, cathepsin G cleaves some platelet surface glycoproteins. As a consequence of platelet activation, cathepsin G increases the binding of antibodies directed at the GpIIbIIIa complex and at the fibrinogen binding site, and decreases the binding of an antibody recognizing the α chain of GpIb, while the binding of an antibody directed at the GpIX is only slightly affected[32–34]. The Leu[275]–Tyr[276] peptide bond has been indicated as the primary cleavage site for this proteinase from N-terminal sequencing of the cathepsin G-derived GpIbα fragment[34]. The increased proteolysis of GpIbα when platelets were inhibited by prostacyclin suggests there may be some cellular redistribution of the GpIbIX receptor during platelet activation by cathepsin G[34]. Actin polymerization and the cytoskeleton play a role in the redistribution of the remaining GpIbIX complex not cleaved by cathepsin G[33].

While the functional significance of the cleavage of GpIbα in the process of platelet activation by cathepsin G remains to be established, it has been clearly shown that this enzyme removes the von Willebrand factor binding site on the GpIb, while leaving the thrombin binding site unaffected. As a consequence, cathepsin G-treated platelets do not agglutinate in response to von Willebrand factor in the presence of ristocetin. Cathepsin G, while activating platelets, might reduce their in vivo reactivity to the subendothelium, through the removal of the von Willebrand factor binding site[32].

Also PMN-derived elastase, which does not cause platelet activation, cleaves GpIb and triggers the exposure of fibrinogen receptors through a proteolytic modification of the GpIIbIIIa[35,36].

Downregulation of platelet function by leukocytes

Early studies showed that the supernatant of apparently unstimulated PMN incubated at 37 °C for 30 minutes contained a platelet inhibitory activity that was compatible with thromboxane-synthase inhibition and/or thromboxane receptor antagonism[37,38]. Thereafter, several in vitro studies have shown that unstimulated PMN could inhibit platelet activation through a NO-like activity[39–43]. This is supported by the elevated levels of platelet cyclic GMP observed in the presence of PMN[40].

In addition to NO-mediated platelet inhibition, human PMN[38,44] and mononuclear leukocytes[45] may also control platelet reactivity via NO-independent mechanisms. Furthermore, unstimulated PMN inhibited activation and recruitment of thrombin- or collagen-stimulated platelets under conditions of close cell–cell contact[46]. The effects were not attributable to NO-like activity, but were modulated by lipoxygenase metabolites and enhanced by aspirin treatment[46]. Platelet ADP removal by ADPase activities expressed by PMN also contributes to down-regulation of platelet activation[47].

Finally, elastase or cathepsin G pretreatment reduces subsequent platelet adhesion to collagen, aggregation and release of granule content induced by thrombin or collagen, through the cleavage of GPIb[48,49].

Activation of leukocytes by platelets and platelet products

Platelet adhesion to the subendothelial matrix of a damaged vessel wall induces platelet activation and the

Cambridge Books Online © Cambridge University Press, 2009

release of substances able to cause the migration of circulating leukocytes toward the sites of injury. Platelet stimulation in whole blood induces oxidative burst in PMN, in conditions of intercellular contact[50]. Supernatants from stimulated platelets are chemotactic for eosinophils[51], induce a transient elevation of PMN intracellular calcium concentration[52], and increase degranulation[53] and superoxide anion generation of stimulated PMN[54,55]. Several platelet-released products endowed with PMN-stimulating activities have been described: Platelet Factor 4 (PF4) and platelet derived growth factor (PDGF), two proteins released by stimulated platelets, as well as arachidonic acid metabolites of the 12-lipoxygenase pathway induce PMN chemotaxis[56–58] and may favour PMN accumulation in vivo. PF4 induces PMN to undergo, through L-selectin and LFA-1, firm adhesion to endothelial cells and to release granule contents[59].

Platelet-dense granules are a relevant source of secreted ATP, which may represent an important mechanism of leukocyte activation: this nucleotide triggers leukocyte intracellular calcium increase and stimulates phosphoinositide breakdown, superoxide anion generation, granule exocytosis[60,61], and the increase of membrane expression of the β2-integrin Mac-1[62]. The identification and tissue distribution of the specific class of purinergic receptors, as well as their cloning and pharmacological characterization have been recently reviewed[63].

A neutrophil-activating peptide, called NAP-2, can be generated from platelet basic protein (PBP) and connective tissue-activating peptide III (CTAP-III), two inactive platelet products, through proteolytic processing operated by leukocyte proteases[64,65]. In addition, thrombin-activated human platelets release two NAP-2 variants, which are not generated outside the platelets by proteolytic processing, but are available already in the active form[66]. Similar results have been reported also with porcine platelets[67].

The synthesis of thrombospondin by monocytes is increased by platelet addition, an effect specific both for the cell type and the protein synthesized[68]. Platelet PF4 induces morphological changes in human monocytes toward a macrophage-like cell type[69], while platelets, or substances shed by activated platelets, enhance macrophage cholesteryl accumulation in conditions of close cell–cell association[70]. Altogether, these observations support the contribution of platelet–monocyte interaction to the progression of atherosclerotic lesion.

Finally, microparticles released from activated platelets enhance both leukocyte aggregation and accumulation of flowing leukocytes on a selectin substrate[71].

Downregulation of leukocytes by platelets

Unstimulated platelets may limit the effects of PMN activation by decreasing their capacity to synthesize PAF[72], to generate oxygen radicals[73], or to produce a cytotoxic effect[74]. Moreover, substances released upon platelet activation, such as TGF-β, the so-called 'adherence inhibiting factor', and P-selectin, which is rapidly lost by activated platelet surface[75], all inhibit PMN adhesion to endothelial cells[76–78]. Soluble P-selectin and PDGF may also reduce the generation of superoxide anion by activated PMN[79,80]. The platelet-derived precursors of NAP-2, CTAP-III and PBP, two CXC chemokines belonging to the subfamily of ELR^+, though lacking PMN-activating properties themselves, are able to down-regulate degranulation and chemotaxis induced by NAP-2 in neutrophils[81,82].

Platelet–monocyte interactions relevant to blood coagulation

A link between platelet–monocyte interactions and blood coagulation has long been established. In particular, generation of a platelet factor X activating activity, and a modulating role in monocyte tissue factor (TF) expression have been proposed.

Semeraro et al.[83], utilizing a system in which platelets were incubated with mononuclear cells in the presence of endotoxin (LPS), observed the development of a platelet coagulant activity of the factor X activator type. Platelets challenged with LPS in the absence of leukocytes were devoid of procoagulant activity, indicating a mediator role for mononuclear cells. Based on these findings a new cellular pathway of blood clotting initiation was proposed.

The first observation of the influence of platelets on mononuclear cell TF was provided by Niemetz and Marcus back in 1974[84]. TF is a 47 kD integral membrane glycoprotein associated with phospholipids, which, upon binding to factor VII and its active form VIIa, triggers blood coagulation leading to fibrin formation[85]. Under normal conditions monocytes do not express TF antigen or activity, but can be induced to do so by exposure to appropriate agonists, among which is LPS[86].

Platelets may influence monocyte TF expression in two different ways: they can induce TF activity in monocytes or enhance TF activity from LPS-stimulated monocytes[84,87,88]. The observation that TF activity correlated with the number of platelets, that arachidonic acid amplified TF expression, and that platelets from donors who had ingested acetylsalicylic acid were more effective, suggested a role for a platelet-derived lipoxygenase pathway metabolite[89]. Indeed, platelet-derived 12-hydroxyeicosatetraenoic

Cambridge Books Online © Cambridge University Press, 2009

acid (12-HETE), the end product of the 12-lipoxygenase-mediated pathway of arachidonic acid, enhanced TF expression in LPS-stimulated mononuclear cells[89]. In addition to 12-HETE, PF4, another platelet-derived mediator, was later reported to increase TF activity in stimulated monocytes[90].

Close contact between platelets and monocytes is required in order to induce TF expression in monocytes (R. Lorenzet, unpublished data), suggesting a role for adhesion molecules, P-selectin being a candidate. An in vivo role for P-selectin in blood clotting activation had been proposed by experiments in a baboon model[91]. The accumulation of leukocytes on activated platelets and fibrin formation, observed within an artificial graft, were greatly reduced by pretreatment of the baboon with anti-P-selectin monoclonal antibodies. This finding indicated that P-selectin in vivo was responsible for platelet–leukocyte binding within the thrombus, and that these leukocytes promoted fibrin formation. Taken together, these observations suggested the possibility that binding of monocytes to activated platelets could affect monocyte procoagulant activity. Indeed, it was shown that purified P-selectin upregulated monocyte TF expression[92]. In addition to P-selectin in the fluid phase, also Chinese hamster ovary cells (CHO) transfected with the P-selectin cDNA, but not with E-selectin, induced TF expression in monocytes.

The inducing effect of P-selectin was later confirmed: in a whole blood model, monocyte TF expression was markedly increased by adding collagen, and was abolished by anti-P-selectin antibodies[93]. A role for P-selectin in mediating the stimulatory effect of platelets and granulocytes on LPS-induced TF activity in monocytes was also shown[94].

12-HETE, similarly to what was observed with LPS-stimulated monocytes, greatly potentiated TF expression by P-selectin-exposed monocytes[95].

Since activated platelets, which express P-selectin on their surface, colocalize with monocytes at sites of vascular injury and thrombosis, signalling for *de novo* monocyte TF synthesis would be important for the maintenance of the local activation of blood coagulation in order to prevent further blood loss.

Lately, it was shown that in mice expressing P-selectin without the cytoplasmic tail an increase in plasma P-selectin was detected; these elevated amounts of plasma P-selectin enhanced the generation of leukocyte-derived microparticles, some of which expressed TF[96].

Finally, a new type of interaction between platelets and leukocytes has been proposed, namely the transfer of TF microparticles from monocytes and, possibly, granulocytes to platelets during thrombus formation[97]. In an in vitro model, the transfer was mediated by TF itself and by the interaction of CD15, the P-selectin counter-receptor (also known as PSGL-1, see below), with platelets. If confirmed, this finding would be of great relevance, since platelets, acquiring TF activity, would be directly capable of triggering and propagating thrombosis.

Transcellular metabolism of arachidonic acid in platelet–leukocyte interaction

The platelet–PMN interaction includes cell–cell co-operation in the arachidonic acid metabolism, which has been defined as transcellular metabolism[3]. Platelets are devoid of 5-lipoxygenase and are thus unable *per se* to synthesise leukotrienes, but platelet-PMN transcellular metabolism of arachidonic acid results in increased leukotriene production, as activated platelets provide free arachidonic acid to activated PMN[98–101]. Platelets may also contribute to LTC4 and lipoxin production by metabolizing PMN-derived LTA4[100,102,103]. PMN, on the other hand, may provide free arachidonic acid to activated platelets, thus increasing the synthesis of cyclooxygenase[104] and 12-lipoxygenase[105] products.

Palmantier and Borgeat[106] first suggested that direct cell–cell contact through specific adhesion molecules may facilitate arachidonic acid transcellular metabolism between platelets and PMN.

Following the observation that L-selectin-mediated PMN–endothelial cell interaction promotes transcellular leukotriene biosynthesis[107], P-selectin-dependent platelet–PMN adhesion has been shown to facilitate the transcellular metabolism of arachidonic acid, resulting in increased production of both TxB2 and LTC4[24].

Adhesive mechanisms

The role of P-selectin and the β2-integrin CD11b/CD18 (Mac-1)

A selective binding of thrombin-stimulated platelets to human monocytes, PMN and related cell lines was shown more than a decade ago[108] and subsequently demonstrated to be primarily mediated by P-selectin[109,110]. P-selectin is a type-1 membrane glycoprotein with a 'C-type' lectin domain, stored in the α-granules of platelets and in the Weibel Palade bodies of endothelial cells (for review see [111,112]). This glycoprotein is exposed on the external surface after cell activation and recognises sialylated fucosylated lactosamines as critical components of specific ligands on different classes of leukocytes. P-selectin glycoprotein ligand-1 (PSGL-1) is a glycoprotein, endowed with high

Cambridge Books Online © Cambridge University Press, 2009

affinity and biologically relevant bonds with P-selectin (for review see [111–114]). In conditions of flow, P-selectin tethers flowing leukocytes and supports subsequent rolling interaction[115], thanks to its ability to bind the PSGL-1 counter-receptor on PMN leukocytes with rapid association and dissociation rates[116,117]. High resolution, high speed imaging under flow allowed direct observation of membrane tethers formed during neutrophil attachment to platelets or P-selectin under physiological flow[118]. Based on the absence of rolling and the severe inflammatory defect reported in E- and P-selectin double-deficient mice[119,120], P-selectin is considered to be an essential requirement for PMN rolling[121]. It is also a prerequisite for β2-integrin-mediated arrest on immobilized platelets following PMN activation by an exogenous chemotactic peptide[122,123].

In a pioneering study performed under physiological shear, Yeo et al.[124] had observed spontaneous conversion from rolling to firm adhesion of PMN on platelets in the absence of exogenous stimuli. Co-operation between P-selectin and the β2-integrin Mac-1 was demonstrated in mixed platelet–PMN suspensions subjected to high speed rotatory motion, a condition emphasizing high affinity adhesive cell-cell interactions not easily disrupted at moderate or high shear forces[125]. In the absence of exogenous stimuli for PMN, activated platelets rapidly formed with them mixed cell conjugates. These were prevented not only, as expected, by anti-P-selectin antibodies, but also by anti-CD18 or anti-CD11b antibodies. Adhesion of pre-activated platelets to PMN resulted in binding to PMN of a monoclonal antibody recognizing an activation and ligand-induced neoepitope on the α-chain of the β2-integrins[126].

More recent observations, obtained in whole blood exposed to hydrodynamic shear comparable to that of venous circulation, indicated that platelet adherence to PMN, via P-selectin binding to PSGL-1, induced Mac-1-dependent platelet–PMN and PMN–PMN aggregation[127]. This confirms the relevance of the molecular mechanisms mentioned above in platelet–PMN interaction in a physiological milieu.

Thus platelets either immobilized on a surface[128] or activated in suspension[125] express a complete machinery to catch flowing PMN: (a) P-selectin mediating the first contact, (b) one or more signalling molecules, relevant for CD11b/CD18 activation and (c) the counter-receptor(s) of the β2-integrin (Fig. 28.3). The latter include the constitutively expressed ICAM-2[129,130], fibrinogen[130,131] bound to GpIIbIIIa[132], GpIbα[133] and probably other not yet characterized receptors[125].

At variance with the adhesive molecules involved in the rolling step that do not require an activation signal to recognize their ligand, β2-integrins require a functional upregulation to become competent to bind their counter-receptor[134]. This implies that an intermediate β2-integrin-activating signal is delivered from the platelet surface to PMN in the few seconds between rolling movement and firm attachment.

To explain this signalling, two possible mechanisms probably cooperating with each other have been proposed[135]: the first assumes that selectin-mediated rolling slows down leukocytes and therefore facilitates PMN and β2-integrin activation by agonists coexpressed with P-selectin on the platelet surface, such as PAF and CXC chemokines[130]. The second possibility is that selectin binding to PMN itself induces a signal enabling β2-integrin adhesiveness.

In the context of the latter hypothesis, Lawrence and Springer[115], using lipid bilayers containing purified selectin and ICAM-1, reported that in flow conditions P-selectin itself does not activate integrins on rolling PMN leukocytes. A similar conclusion was reached by Lorant et al.[136], showing that although soluble P-selectin does not directly induce β2-integrin adhesiveness, it potentiates the β2-integrin-dependent adhesive response to PAF. More recently, however, evidence was provided supporting a direct role for P-selectin in influencing β2-integrin function: indeed purified P-selectin was able to increase the PMN phagocytosis of unopsonized zymosan, which is a recognized Mac-1-dependent function[137], and to stimulate Mac-1-dependent homotypic aggregation of human PMN[138] and LFA-1-, Mac-1-dependent adhesion of mouse PMN to ICAM-1[139].

The role of PSGL-1 and of protein tyrosine phosphorylation as signalling molecules

As already mentioned, an important role in transmitting the P-selectin-induced signal is played by PSGL-1[138,139]; engagement of PSGL-1 by P-selectin or by specific monoclonal antibodies induces protein tyrosine phosphorylation[138,140] and activates mitogen-activated protein (MAP) kinases in human PMN leukocytes[140]. Consistently, tyrosine kinase inhibitors prevented Mac-1 adhesion[125], as well as IL-8 production triggered by P-selectin or PSGL-1 engagement by monoclonal antibodies.

Among different families of protein tyrosine kinases present in leukocytes, those belonging to the family of SRC play a major role in β2-integrin adhesive function triggered by P-selectin[141]. Specific inhibitors of SRC tyrosine kinases were found to inhibit PMN adhesion to activated platelets or to P-selectin expressing CHO (CHO-P) cells as well as protein tyrosine phosphorylation in PMN. Adhesion of

Cambridge Books Online © Cambridge University Press, 2009

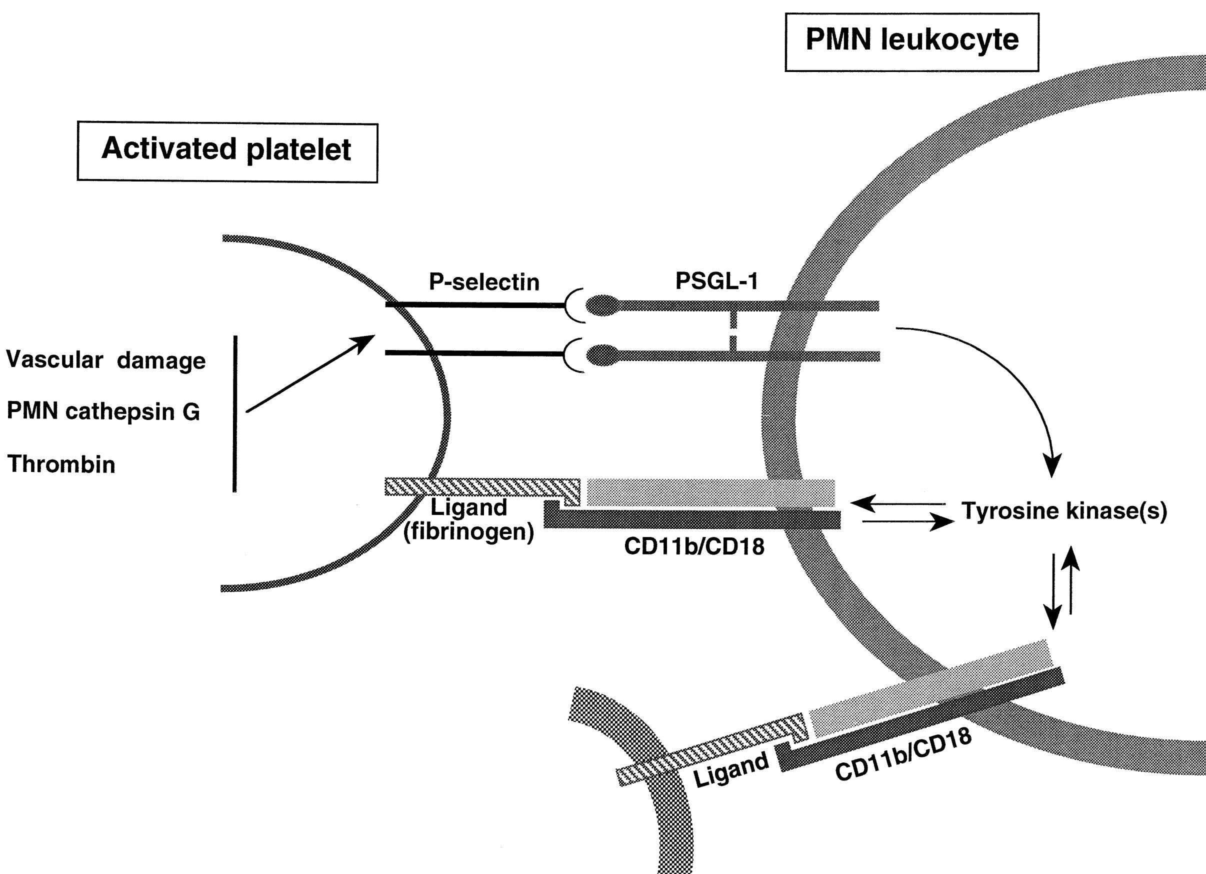


Fig. 28.3. Schematic sequence of the molecular events following platelet activation by different means. P-selectin expressed on activated platelet surface binds to PSGL-1 on PMN leukocytes, thus establishing a rapidly reversible tethering. PSGL-1 engagement starts a signalling mechanism, involving protein tyrosine phosphorylation. The latter is followed by activation of the β2-integrin Mac-1, which becomes able to bind to platelet counter-receptors (including fibrinogen) or to other β2-integrin ligands that allow multicellular interactions, as in Fig. 28.7. At this moment the interaction between platelets and PMN leukocytes becomes stable. (This figure is reprinted[8], with permission.)

PMN to activated platelets or to CHO-P cells stimulated the activity of LYN and HCK, two SRC kinases, while monoclonal antibody blockade of P-selectin or β2-integrins reduced the activation of both kinases. In PMN, either adherent to platelets or aggregated by P-selectin-IgG chimera or by anti-PSGL-1 antibodies, Mac-1 was rapidly redistributed to the Triton X-100-insoluble cytoskeletal fraction and large clusters of this β2-integrin colocalized with patches of F-actin at the site of cell-cell contact[141] (Fig. 28.4, see colour plate). In PMN stimulated by P-selectin-IgG chimera, SRC kinase inhibition impaired Mac-1 clustering and F-actin accumulation and CD18 redistribution to the cytoskeleton. Conversely, disruption of actin filament network by cytochalasin D prevented PMN-platelet adhesion and P-

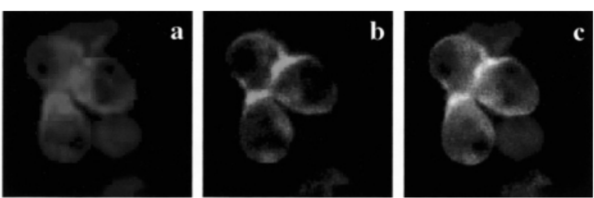


Fig. 28.4 (see also colour plate). Formation of Mac-1 clusters and colocalization with F-actin patches in PMN leukocytes at the site of cell–cell contact. Confocal laser scanning microscopy of PMN, preincubated with an anti-PSGL-1 antibody, stained with FITC-conjugated anti-CD11b antibody and rodamine-phalloidin: (*a*) and (*b*) show F-actin (red) and CD11b (green) staining, respectively. In the overlay (*c*), the yellow colour represents colocalization of the two stainings. (For more details[141].)

Cambridge Books Online © Cambridge University Press, 2009

selectin-induced PMN aggregation and impaired clustering of Mac-1. In agreement with the requirement for the β2-integrin in the functional upregulation of LYN and HCK, integrin blockade by monoclonal antibodies resulted in a complete inhibition of Mac-1 clustering and F-actin accumulation in P-selectin-challenged PMN[141]. These results indicate that, after the initial β2-integrin interaction with its ligand, SRC kinases are activated and allow the remodelling of cytoskeleton–β2-integrin linkages and clustering that finally strengthen cell–cell adhesion. This model highlights a new role for SRC kinases in a regulatory loop by which Mac-1 promotes its own adhesive function, but leaves open the question about the initial signal triggered by P-selectin that upregulates β2-integrin[141].

The contributory role of platelet–leukocyte interaction in vascular damage and thrombosis

Evidence from experimental animal models

Evidence of platelet–leukocyte interaction and leukocyte incorporation into and/or onto thrombi has been found in different experimental models (for reviews see refs[5,7,9]). On the one hand, thrombocytopenia decreases leukocyte recruitment after vascular injury in a porcine model[142], on the other platelet accumulation in experimental myocardial infarction is reduced by PMN depletion[143]. Activated PMN produce and release reactive oxygen species, inflammatory leukotrienes and proteolytic lysosomal enzymes, which can directly induce vascular damage and influence the activation state of both platelets and endothelial cells[7]. In animal models of myocardial infarction, PMN depletion, pharmacological suppression of PMN activation, as well as inhibition of PMN–endothelial cell adhesion reduced the extent of acute tissue injury and mortality following ischemia and reperfusion[143–146].

Mixed masses of PMN and platelets have been observed in the lungs of experimental animals subjected to hemorrhagic shock[147] or anaphylatoxin instillation[148] or following intradermal or intravenous infusion of fMLP[149,150], or in rat glomeruli in a model of immune complex nephritis[151].

The classical observations of leukocyte accumulation at the site of platelet thrombus have already been mentioned[1,91]. Platelet–leukocyte interaction in circulating blood was also observed after injection of oxidised lipoproteins in vivo[152]. When native whole blood was perfused over a collagen-coated surface, the platelet thrombi were coated with a layer of leukocytes[153]. The pathogenesis of the adult respiratory distress syndrome also appears to involve activation of both platelets and PMN[154]. Platelets and PMN leukocytes act synergistically in provoking post-reperfusion cardiac dysfunction in rats[155] or vasoconstriction after deep carotid arterial injury by angioplasty in pigs[156].

The transcellular biosynthesis of sulphidopeptide leukotrienes is accompanied by morphological changes and coronary vasospasm in isolated rabbit heart preparations perfused with activated human PMN[157]. Pretreatment with leukotriene synthesis inhibitors prevented morphological alterations and protected against the increase in coronary perfusion pressure[157,158]. In experiments of permanent coronary artery ligature in vivo a leukotriene synthesis inhibitor reduced the mortality rate and protected the rabbits against the marked electrocardiogram derangement induced by ligation[158]. A monoclonal anti-CD18 antibody prevented transcellular biosynthesis of cysteinyl leukotrienes in vitro and in vivo and protected against LT-dependent increase in coronary resistance[159].

Intravenous infusion of the inflammatory chemotactic agent fMLP in the rabbit induced a dramatic fall in circulating leukocytes and platelets; the latter were activated and aggregated together with leukocytes in the capillaries and venules of the lungs[150]. The concomitant synthesis of eicosanoids and ischemic electrocardiographic changes were prevented by treatment with thromboxane inhibitors, supporting platelet involvement in this model of primary leukocyte activation[160,161] (Fig. 28.5).

Altogether these data provide experimental support to the hypothesis that inflammation is an intermediate mechanism relevant in acute coronary syndromes that can be sustained by platelet–leukocyte interaction.

Experimental evidence also supports the contribution of platelet–leukocyte interactions to the intimal hyperplasia, caused by vascular injury. Transluminal wire injury to the mouse femoral artery induces abundant intimal hyperplasia formation within 2–4 weeks, but already one hour after injury the denuded surface was covered with platelets and leukocytes, predominantly neutrophils, and associated with the accumulation of the adhesion molecules P-selectin, VCAM-1 and ICAM-1[162]. In a balloon-injury model of rat carotid artery, inhibition of P-selectin-mediated leukocyte recruitment prevented the development of neointimal formation, adventitial inflammation, and vascular shrinking, and promoted pseudo-endothelialization by luminal smooth muscle cells[163]. P-selectin-deficient mice are protected from developing intimal hyperplasia after ligating the carotid artery[164], and have defects in leukocyte rolling in the absence of inflammation and in the early recruitment of PMN in the presence of inflammation[165]. P-selectin-deficient mice also show significant protection from reperfusion injury in different vascular districts[166–171].

Cambridge Books Online © Cambridge University Press, 2009

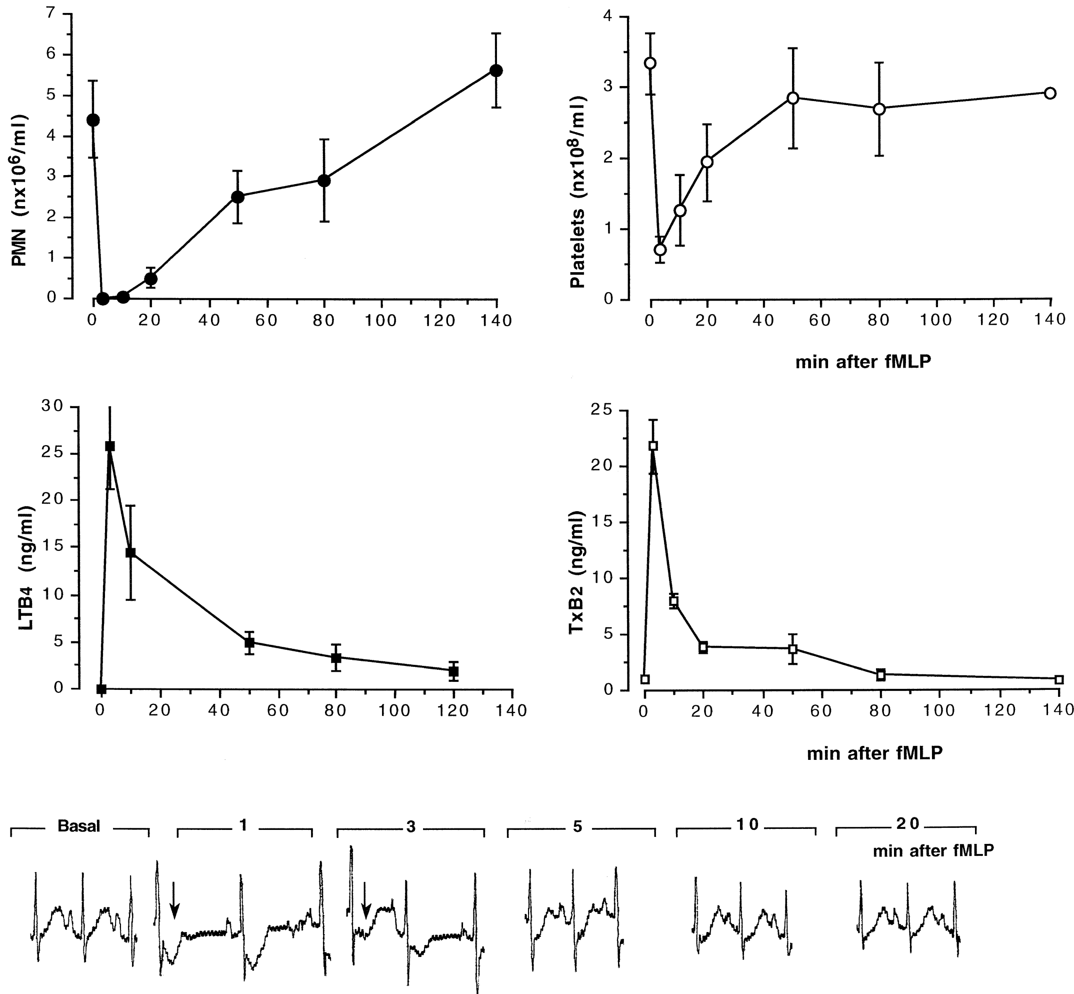


Fig. 28.5. Effect of intravenous infusion of fMLP in rabbits on PMN and platelet count, eicosanoid production (LTB4 and TxB2) and an electrocardiographic tracing showing signs of acute reversible ischemia (indicated by the arrows). (For more details[160,161].)

A recent study tested the hypothesis that the absence of either P-selectin or ICAM-1 inhibits macrophage recruitment into the vessel wall and protects against neointimal formation after arterial denudation in atherosclerosis-prone apolipoprotein E-deficient mice. The results indicate that the complete absence of P-selectin, but not ICAM-1, markedly reduces plaque area and suggest that P-selectin is critical for monocyte recruitment to sites of neointima formation after arterial injury[172].

In addition, anti-Mac-1 antibodies inhibited intimal hyperplasia in a rabbit model[173], and Mac-1-deficient mice were protected from developing neointimal thickening following injury to the carotid vasculature[174]. The role of P-selectin in the development of intimal hyperplasia was recently confirmed in P-selectin-deficient mice after endothelial injury; interestingly enough, β3-integrin-deficient mice were not protected[175].

Most recently, infiltration of PMN into the media of blood vessels was observed in the rabbit within a few hours after injury, and a correlation was found between early PMN transmigration and subsequent smooth muscle cell proliferation[176].

From these experimental studies it appears that P-selectin and Mac-1 are the major mediators responsible for PMN recruitment in an injured vessel covered by platelets.

Evidence from epidemiological and clinical studies

Epidemiological evidence suggests a positive correlation between the number of leukocytes, PMN in particular, and the risk of ischemic vascular disease (for review see [177–179]). Indeed high PMN count was associated with an increased risk of acute myocardial infarction[177] and its recurrence[180] or of transient cerebral ischemic attacks[181]. Further studies

Cambridge Books Online © Cambridge University Press, 2009

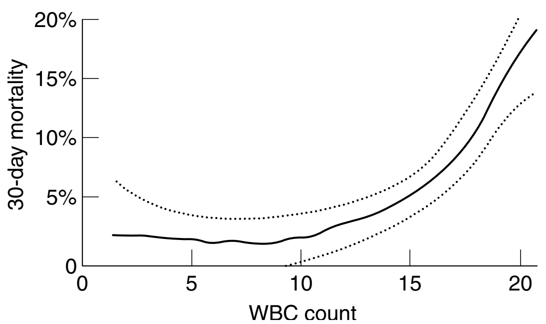


Fig. 28.6. Association of baseline white blood cell count ($\times 10^3/\mu l$) with increased 30-day mortality in acute myocardial infarction and unstable angina patients. (This figure is reprinted[184], with permission from Excerpta Medica Inc.)

reported increased ex vivo functional responsiveness as well as in vivo PMN activation and platelet–leukocyte interaction in different clinical manifestations of ischemic heart disease and suggested an active role for these cells in the progression of vascular occlusion[182,183]. Recently, association of white blood cell count with increased mortality in acute myocardial infarction and unstable angina has been reported[184] (Fig. 28.6).

Elevated levels of soluble P-selectin have been found in atherosclerotic, hyperlipidemic and hypertensive patients[185–190]. Recently, elevated baseline levels of soluble P-selectin were found to be associated with increasing risk of future myocardial infarction, stroke, coronary revascularization and cardiovascular death in a large scale prospective study of apparently healthy women. This association was independent of traditional risk factors[191].

Although most platelet–leukocyte aggregates have probably a short intravascular survival time, so that only a small fraction of the formed aggregates are present in the systemic circulation at any time, platelet–leukocyte conjugates have been observed in peripheral blood from patients with unstable angina[192]. Cell–cell interaction might trigger PMN activation in this clinical condition[182], as originally reported by Mazzone et al[183]. On the other hand, the formation of platelet–PMN conjugates following percutaneous coronary interventions was considered a predictive index of acute reocclusion[192,193]. Circulating monocyte–platelet aggregates have been identified in patients with stable coronary artery disease[194].

Activated leukocytes, as judged by increased surface expression of Mac-1, have been reported in the systemic circulation of patients admitted to hospital with chest pain[195], in patients after myocardial infarction[196] or after coronary angioplasty[197] and in the coronary sinus blood of patients with unstable (but not stable) angina[183,198]. On the other hand, monocytes with attached platelets from patients undergoing stenting for acute myocardial infarction express more Mac-1 than monocytes without attached platelets[199]. In patients with unstable angina, increased Mac-1 expression was measured on leukocytes from coronary sinus blood compared to blood from either the coronary ostium or the coronary artery just distal to the culprit lesion, suggesting that leukocyte activation occurs within the microcirculation of the myocardium[200].

Induction of cytokine expression in PMN leukocytes by binding of thrombin-stimulated platelets was observed in patients with unstable angina or myocardial infarction[201].

Pharmacology of the platelet–leukocyte interactions

As the intensity of the platelet response is directly related to the degree of PMN activation, substances inhibiting PMN stimulation may prevent the platelet–PMN interaction. This is the case with serine proteinase inhibitors such as α1-proteinase inhibitor, secretory leukocyte proteinase inhibitor, or its truncated form, and eglin C[202–204]. However, cathepsin G discharged from stimulated PMN on strictly adherent platelets may be protected from these antiproteinases[21,205].

Heparin, one of the most widely used drugs for prevention and treatment of thrombosis, has three effects which may be relevant to pharmacological modulation of the platelet–PMN interaction. First, heparin may interfere with P-selectin-dependent platelet–PMN adhesion[206]; secondly, it prevents platelet activation induced by stimulated PMN by acting on cathepsin G, possibly through an ionic interaction between differently charged molecules[203,207,208]. Heparin, as an adhesive ligand for Mac-1[209,210], may interfere with cell adhesion, thus avoiding the formation of a protected microenvironment, at the same time preventing the effect of cathepsin G on platelets. The effect of heparin, independent of its polyanionic and its anticoagulant activity has been recently confirmed by studying the adhesion of human PMN to vascular endothelium[211]. Other glycosaminoglycans, such as sulodexide, low-molecular-weight dermatansulfate and heparins with different degrees and types of sulfation have also been shown to inhibit platelet aggregation induced by cathepsin G and its catalytic activity[212]. Thirdly, heparin and other glycosaminoglycans inhibited lysosomal enzyme release and superoxide production by activated PMN[208,212]. In the already mentioned rabbit model of stent-induced arterial injury, heparin was shown to inhibit early neutrophil infiltration in the media, smooth muscle cell proliferation and neointimal growth at 7–14 days[176].

These observations may help establish whether

Cambridge Books Online © Cambridge University Press, 2009

mechanism(s) of action other than anticoagulation may contribute to the antithrombotic activity of heparin and related compounds. Like heparin, defibrotide, a polydeoxyribonucleotide with antithrombotic effects in experimental models, inhibits platelet activation by stimulated PMN, through inactivation of cathepsin G catalytic activity. Defibrotide is inactive on thrombin[213].

Several compounds known to affect platelet response have also been tested for their ability to modify platelet–PMN interaction in vitro. As already observed on platelets challenged with thrombin, aspirin, even at high concentrations, did not prevent platelet activation induced by PMN or cathepsin G, although it completely suppressed TxB2 generation[19,20,25]. Thus aspirin treatment of patients with ischemic events may not reduce platelet activation at sites of PMN accumulation such as myocardial infarction areas. The reported efficacy of dipyridamole in preventing PMN-dependent platelet activation, as well as PMN oxygen radical generation when triggered by LTB4[20,214] is of interest, but remains to be confirmed.

Iloprost, a stable prostacyclin analogue, appears to be active at nanomolar concentrations on PMN–platelet interaction and at micromolar concentrations on PMN activation. Iloprost reportedly helped the healing of cutaneous ulcers and reduced amputation in patients with critical leg ischemia, where leukocyte–platelet activation may have pathogenic relevance[215].

While β-adrenergic blocking agents appear to enhance leukocyte function, no data are available on a possible consequent increase of platelet activation. Similarly, it is not known whether the inhibitory effect of calcium antagonists and nitrates on PMN function has any consequence on platelet–PMN interaction[216].

Preliminary data show that in patients undergoing coronary interventions, treatment with abciximab, an antiGpIIbIIIa chimeric monoclonal antibody, not only prevents the increase in PMN and monocyte Mac-1 expression that occurs postangioplasty, but actually results in an absolute decrease in Mac-1 expression on both cell types[197]. A study in which human platelets, leukocytes, and fibrinogen were infused into isolated guinea pig hearts after 15 min of ischemia and 2 min of reperfusion, indicated that abciximab treatment not only prevented the transcoronary increase in neutrophil Mac-1 and the development of platelet-PMN aggregates, but also facilitated postischemic recovery[217]. A recent study in patients undergoing coronary angioplasty[218] demonstrates that, although abciximab treatment decreases the percentage of circulating platelet–leukocyte aggregates, it promotes P-selectin-mediated platelet–leukocyte interaction on a collagen-von Willebrand factor surface. On the other hand, platelet–monocyte interaction decreased after abciximab treatment in patients with acute myocardial infarction, mainly due to a reduction in platelet mass attached to monocytes, as it did not affect the percentage of monocytes with adherent platelets[199].

One can speculate that drugs interfering with specific platelet–leukocyte interaction mechanism(s), would block both platelet and leukocyte contribution to thrombus formation and might prove to be clinically more effective than drugs only affecting either cell type[9]. Although platelet GpIIbIIIa might be indirectly involved in platelet–leukocyte interaction mediated by fibrinogen, compounds directly inhibiting the function of P-selectin, β2-integrin or their respective counter-receptors should be identified and developed for experimental and clinical testing.

Following the already mentioned observation[91] that platelet P-selectin-dependent leukocyte accumulation in an experimental thrombosis model in the baboon promotes fibrin deposition, it has been shown that P-selectin blockade by specific monoclonal antibodies[219] or with a soluble recombinant form of PSGL-1[220] accelerates the pharmacological lysis of arterial thrombi and prevents reocclusion in animal models. P-selectin blockade also reduces PMN adhesion to platelets at the site of a deep injury in the carotid artery of pigs[221] and reduces cyclic occlusion in a canine model of recurrent arterial thrombosis[222]. P-selectin antagonism with recombinant soluble PSGL-1 inhibits early platelet–leukocyte adhesion on pig injured arteries and reduces restenosis after angioplasty through a positive impact on vascular remodelling[223].

Compounds interfering with the intracellular signalling mechanisms, including the control of tyrosine kinases, might also be of some pharmacological and therapeutic relevance. In this context, it is of interest to mention that genistein, piceatannol and other relatively specific inhibitors of leukocyte tyrosine kinases have been found in naturally derived nutrients and/or in wine[224]. And wine is presently considered as a dietary means to prevent ischemic heart disease[225,226]. *Trans*-resveratrol, a polyphenolic compound also contained in red wine, prevents platelet–PMN adhesion and Mac-1 expression by PMN in vitro[227].

In conclusion, inflammation plays a major role in atherogenesis and thrombosis[228]. While the role of platelets in these phenomena was recognized by many investigators over the last century, the involvement of leukocytes, major players in inflammation, was only appreciated in the last two decades[229].

Slowing, tethering and subsequent attachment of circulating leukocytes to the vascular endothelium is presently considered among the earliest inflammatory steps leading

Cambridge Books Online © Cambridge University Press, 2009

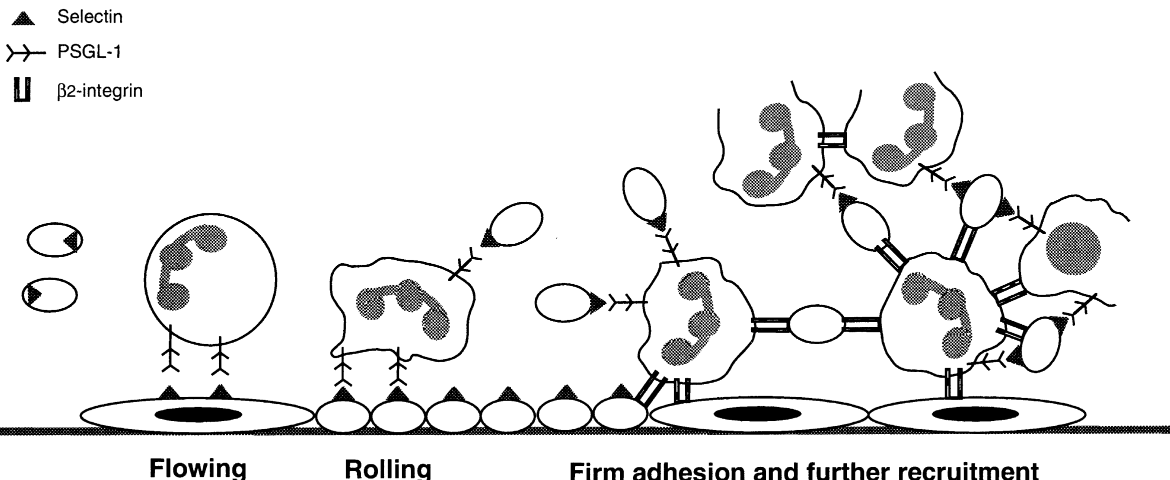


Fig. 28.7. Hypothetical sequence of the interactions between PMN leukocytes and activated platelets or injured endothelial cells. Binding of P-selectin (or E-selectin) to PSGL-1 promotes tethering and rolling of PMN leukocytes on vascular surface. PSGL-1-induced activation of PMN β2-integrin(s) allows stable multicellular interactions. Cathepsin G released by activated PMN leukocytes may facilitate further platelet and PMN recruitment at the vascular injury site. (This figure is reprinted[8], with permission.)

to vascular occlusion[228]. In this process the possible contribution of platelet–leukocyte interactions has been progressively characterized[7,8], as well as the importance of the selectin and integrin families of cell adhesion molecules[230–232] (Fig. 28.7). Basic work in vitro and in experimental models suggests that platelet–leukocyte cross-talk plays an important role in the initiation and progression of athero-thrombosis.

Clinical and epidemiological data, although relatively limited, already provide confirmation of the cardiovascular risk associated with cell–cell interaction and related molecular adhesive mechanisms[191].

Finally, it is likely that new pharmacological avenues will be scoured to develop new preventive and therapeutic approaches based on the cellular and molecular pathways described in this chapter.

Acknowledgements

Marina Molino, Paola Piccardoni, Serenella Rotondo, Rita Sideri, Licia Totani, Antonio Celardo, Giuseppe Dell'Elba, Angelomaria Di Santo, Stefano Manarini, Nicola Martelli, Emanuela Napoleone, Antonio Piccoli contributed to the authors' experimental work mentioned in this chapter.

Mrs Filomena Cinalli and Maria Grazia Mencuccini helped prepare the manuscript.

The support of the Italian National Research Council (Convenzione CNR–Consorzio Mario Negri Sud) is acknowledged.

References

1 Bizzozero J. Uber einen neuen formbestandteil des blutes und dessen rolle bei der thrombose und blutgerinnung. *Virchows Arch Pathol Anat Physiol Klin Med* 1882; 90: 261–332.
2 Weksler BB. Roles for human platelets in inflammation. *Prog Clin Biol Res* 1988; 283: 611–638.
3 Marcus AJ. Thrombosis and inflammation as multicellular processes: pathophysiologic significance of transcellular metabolism. *Blood* 1990; 76: 1903–1907.
4 de Gaetano G, Evangelista V, Rajtar G, Del Maschio A, Cerletti C. Activated polymorphonuclear leukocytes stimulate platelet function. *Thromb Res Suppl* 1990; 11: 25–32.
5 Bazzoni G, Dejana E, Del Maschio A. Platelet–neutrophil interactions. Possible relevance in the pathogenesis of thrombosis and inflammation. *Haematologica* 1991; 76: 491–499.
6 Cerletti C, Evangelista V, de Gaetano G. Polymorphonuclear leucocyte-dependent modulation of platelet function: relevance to the pathogenesis of thrombosis. *Pharmacol Res* 1992; 26: 261–268.
7 Cerletti C, Evangelista V, Molino M, de Gaetano G. Platelet activation by polymorphonuclear leukocytes: role of cathepsin G and P-selectin. *Thromb Haemost* 1995; 74: 218–223.
8 Cerletti C, Evangelista V, de Gaetano G. P-selectin-beta 2-integrin cross-talk: a molecular mechanism for polymorphonuclear leukocyte recruitment at the site of vascular damage. *Thromb Haemost* 1999; 82: 787–793.
9 Coller BS. Binding of abciximab to alpha V beta 3 and activated alpha M beta 2 receptors: with a review of platelet-leukocyte interactions. *Thromb Haemost* 1999; 82: 326–336.

Cambridge Books Online © Cambridge University Press, 2009

10 Handin RI, Karabin R, Boxer GJ. Enhancement of platelet function by superoxide anion. *J Clin Invest* 1977; 59: 959–965.
11 Mehta P, Mehta J, Lawson D, Krop I, Letts LG. Leukotrienes potentiate the effects of epinephrine and thrombin on human platelet aggregation. *Thromb Res* 1986; 41: 731–738.
12 Chignard M, Kerali CL, Nuñez D, Cöeffier E, Benveniste J. PAF-acether and platelets. In MacIntyre DE, Gordon JL, eds. *Platelets in biology and patholog*y III. Cambridge: Elsevier Science Publishers: 1987; pp 289–315.
13 Li N, Hu H, Lindqvist M, Wikstrom-Jonsson E, Goodall AH, Hjemdahl P. Platelet–leukocyte cross talk in whole blood. *Arterioscler Thromb Vasc Biol* 2000; 20: 2702–2708.
14 Altieri DC, Mannucci PM. Thromboxane generation by human monocytes enhances platelet function. *J Exp Med* 1986; 164: 1815–1820.
15 Lazzari MA, Schattner MA, Finiasz M, Gimeno M. Platelet aggregating substance from mononuclear leukocytes. *Prostaglandins Leukot Med* 1986; 22: 275–284.
16 Schwartz BS, Monroe MC. Human platelet aggregation is initiated by peripheral blood mononuclear cells exposed to bacterial lipopolysaccharide in vitro. *J Clin Invest* 1986; 78: 1136–1141.
17 Hällgren R, Venge P. Cationic proteins of human granulocytes. Effects on human platelet aggregation and serotonin release. *Inflammation* 1976; 1: 359–370.
18 Bykowska K, Kaczanowska J, Karpowicz M, Stachurska J, Kopec´ M. Effect of neutral proteases from blood leukocytes on human platelets. *Thromb Haemost* 1983; 50: 768–772.
19 Chignard M, Selak MA, Smith JB. Direct evidence for the existence of a neutrophil-derived platelet activator (neutrophilin). *Proc Natl Acad Sci USA* 1986; 83: 8609–8613.
20 Del Maschio A, Evangelista V, Rajtar G, Chen ZM, Cerletti C, de Gaetano G. Platelet activation by polymorphonuclear leukocytes exposed to chemotactic agents. *Am J Physiol* 1990; 258: H870-H879.
21 Evangelista V, Rajtar G, de Gaetano G, White JG, Cerletti C. Platelet activation by fMLP-stimulated polymorphonuclear leukocytes: the activity of cathepsin G is not prevented by antiproteinases. *Blood* 1991; 77: 2379–2388.
22 Selak MA, Chignard M, Smith JB. Cathepsin G is a strong platelet agonist released by neutrophils. *Biochem J* 1988; 251: 293–299.
23 Selak MA. Neutrophil elastase potentiates cathepsin G-induced platelet activation. *Thromb Haemost* 1992; 68: 570–576.
24 Maugeri N, Evangelista V, Celardo A et al. Polymorphonuclear leukocyte-platelet interaction: role of P-selectin in thromboxane B_2 and leukotriene C_4 cooperative synthesis. *Thromb Haemost* 1994; 72: 450–456.
25 Molino M, Di Lallo M, de Gaetano G, Cerletti C. Intracellular Ca^{2+} rise in human platelets induced by polymorphonuclear-leucocyte-derived cathepsin G. *Biochem J* 1992; 288: 741–745.
26 Rotondo S, Evangelista V, Manarini S, de Gaetano G, Cerletti C. Different requirement of intracellular calcium and protein kinase C for arachidonic acid release and serotonin secretion in cathepsin G-activated platelets. *Thromb Haemost* 1997; 78: 919–925.
27 Selak MA, Smith JB. Cathepsin G binding to human platelets. Evidence for a specific receptor. *Biochem J* 1990; 266: 55–62.
28 Vu TKD, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* 1991; 64: 1057–1068.
29 Selak MA. Cathepsin G and thrombin: evidence for two different platelet receptors. *Biochem J* 1994; 297: 269–275.
30 Molino M, Blanchard N, Belmonte E et al. Proteolysis of the human platelet and endothelial cell thrombin receptor by neutrophil-derived cathepsin G. *J Biol Chem* 1995; 270: 11168–11175.
31 Sambrano GR, Huang W, Faruqi T, Mahrus S, Craik C, Coughlin SR. Cathepsin G activates protease-activated receptor-4 in human platelets. *J Biol Chem* 2000; 275: 6819–6823.
32 Molino M, Di Lallo M, Martelli N, de Gaetano G, Cerletti C. Effects of leukocyte-derived cathepsin G on platelet membrane glycoprotein Ib–IX and IIb–IIIa complexes: a comparison with thrombin. *Blood* 1993; 82: 2442–2451.
33 LaRosa CA, Rohrer MJ, Benoit SE, Barnard MR, Michelson AD. Neutrophil cathepsin G modulates the platelet surface expression of the glycoprotein (GP) Ib–IX complex by proteolysis of the von Willebrand factor binding site on GPIbα and by a cytoskeletal-mediated redistribution of the remainder of the complex. *Blood* 1994; 84: 158–168.
34 Pidard D, Renesto P, Berndt MC, Rabhi S, Clemetson KJ, Chignard M. Neutrophil proteinase cathepsin G is proteolytically active on the human platelet glycoprotein Ib-IX receptor: characterization of the cleavage sites within the glycoprotein Ibα subunit. *Biochem J* 1994; 303: 489–498.
35 Kornecki E, Ehrlich YH, Egbring R et al. Granulocyte-platelet interactions and platelet fibrinogen receptor exposure. *Am J Physiol* 1988; 255: H651-H658.
36 Aziz KA, Cawley JC, Kamiguti AS, Zuzel M. Degradation of platelet glycoprotein Ib by elastase released from primed neutrophils. *Br J Haematol* 1995; 91: 46–54.
37 Villa S, Rotilio D, Donati MB, de Gaetano G. Human blood leucocytes in vitro generate an inhibitor of platelet aggregation. *Thromb Res* 1981; 24: 485–487.
38 Zatta A, Prosdocimi M, Bertelé V, Bazzoni G, Del Maschio A. Inhibition of platelet function by polymorphonuclear leukocytes. *J Lab Clin Med* 1990; 116: 651–660.
39 Rimele TJ, Sturm RJ, Adams LM et al. Interaction of neutrophils with vascular smooth muscle: identification of a neutrophil-derived relaxing factor. *J Pharmacol Exp Ther* 1988; 245: 102–111.
40 Salvemini D, deNucci G, Gryglewski RJ, Vane JR. Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor. *Proc Natl Acad Sci USA* 1989; 86: 6328–6332.
41 McCall TB, Boughton-Smith NK, Palmer RM, Whittle BJ, Moncada S. Synthesis of nitric oxide from L-arginine by neu-

Cambridge Books Online © Cambridge University Press, 2009

trophils. Release and interaction with superoxide anion. *Biochem J* 1989; 261: 293–296.

42 Nicolini FA, Wilson AC, Mehta P, Mehta JL. Comparative platelet inhibitory effects of human neutrophils and lymphocytes. *J Lab Clin Med* 1990; 116: 147–152.

43 Faint RW, Mackie IJ, Machin SJ. Platelet aggregation is inhibited by a nitric oxide-like factor released from human neutrophils in vitro. *Br J Haematol* 1991; 77: 539–545.

44 Schattner MA, Geffner JR, Isturiz MA, Lazzari MA. Inhibition of human platelet activation by polymorphonuclear leukocytes. *Br J Pharmacol* 1990; 101: 253–256.

45 Schattner MA, Finiasz MR, Notrica JA, Lazzari MA. Platelet aggregation inhibition by mononuclear leukocytes. *Thromb Res* 1994; 73: 205–214.

46 Valles J, Santos MT, Marcus AJ et al. Downregulation of human platelet reactivity by neutrophils. Participation of lipoxygenase derivatives and adhesive proteins. *J Clin Invest* 1993; 92: 1357–1365.

47 Zatta A, Pandolfo L, Caparrotta L, Prosdocimi M, Dejana E, Del Maschio A. Platelet aggregation induced by the endoperoxide analogue U46619 is inhibited by polymorphonuclear leukocyte ADPase activity. *Arterioscler Thromb* 1993; 13: 696–701.

48 Wicki AN, Clemetson KJ. Structure and function of platelet membrane glycoproteins Ib and V. Effects of leukocyte elastase and other proteases on platelets response to von Willebrand factor and thrombin. *Eur J Biochem* 1985; 153: 1–11.

49 Kinlough-Rathbone RL, Perry DW, Rand ML, Packham MA. Effects of cathepsin G pretreatment of platelets on their subsequent responses to aggregating agents. *Thromb Res* 1999; 95: 315–323.

50 Ruf A, Schlenk RF, Maras A, Morgenstern E, Patscheke H. Contact-induced neutrophil activation by platelets in human cell suspensions and whole blood. *Blood* 1992; 80: 1238–1246.

51 Burgers JA, Schweizer RC, Koenderman L, Bruijnzeel PLB, Akkerman JWN. Human platelets secrete chemotactic activity for eosinophils. *Blood* 1993; 81: 49–55.

52 Takahashi A, Yamamoto K, Okuma M, Sasada M. Transient calcium elevation in polymorphonuclear leukocytes triggered by thrombin-activated platelets. *Eur J Haematol* 1992; 48: 196–201.

53 Del Maschio A, Corvazier E, Maillet F, Kazatchkine MD, Maclouf J. Platelet-dependent induction and amplification of polymorphonuclear leucocyte lysosomal enzyme release. *Br J Haematol* 1989; 72: 329–335.

54 Ward PA, Cunningham TW, McCulloch KK, Phan SH, Powell J, Johnson KJ. Platelet enhancement of O_2^- responses in stimulated human neutrophils. Identification of platelet factor as adenine nucleotide. *Lab Invest* 1988; 58: 37–47.

55 Colli S, Eligini S, Lalli M, Tremoli E. Platelet-neutrophil interaction and superoxide anion generation: involvement of purine nucleotides. *Free Radic Biol Med* 1996; 20: 271–278.

56 Deuel TF, Senior RM, Chang D, Griffin GL, Heinrikson RL, Kaiser ET. Platelet factor 4 is chemotactic for neutrophils and monocytes. *Proc Natl Acad Sci USA* 1981; 78: 4584–4587.

57 Deuel TF, Senior RM, Huang JS, Griffin GL. Chemotaxis of monocytes and neutrophils to platelet-derived growth factor. *J Clin Invest* 1982; 69: 1046–1049.

58 Goetzl EJ, Woods JM, Gorman RR. Stimulation of human eosinophil and neutrophil polymorphonuclear leukocyte chemotaxis and random migration by 12–L-hydroxy-5,8,10,14-eicosatetraenoic acid. *J Clin Invest* 1977; 59: 179–183.

59 Peterson F, Boch L, Flad HD, Brandt E. Platelet Factor 4-induced neutrophil-endothelial cell interaction: involvement of mechanisms and functional consequences different from those elicited by interleukin-8. *Blood* 1999; 94: 4020–4028.

60 Cockcroft S, Stutchfield J. ATP stimulates secretion in human neutrophils and HL60 cells via a pertussis toxin-sensitive guanine nucleotide-binding protein coupled to phospholipase C. *FEBS Lett* 1989; 245: 25–29.

61 Balazovich KJ, Boxer LA. Extracellular adenosine nucleotides stimulate protein kinase C activity. *J Immunol* 1990; 144: 631–637.

62 Freyer DR, Boxer LA, Axtell RA, Todd III RF. Stimulation of human neutrophil adhesive properties by adenine nucleotides. *J Immunol* 1988; 141: 580–586.

63 Di Virgilio F, Chiozzi P, Ferrari D et al. Nucleotide receptors: an emerging family of regulatory molecules in blood. *Blood* 2001; 97: 587–600.

64 Walz A, Dewald B, Baggiolini M. Formation and biological activity of NAP-2, a neutrophil-activating peptide derived from platelet alpha-granule precursors. In Oppenheim JJ, Powanda MC, Kluger MJ, Dinarello CA, eds. *Molecular and cellular biology of cytokines*. New York: Wiley-Liss; 1990: 363–368.

65 Yan Z, Zhang J, Holt JC, Stewart GJ, Niewiarowski S, Poncz M. Structural requirements of platelet chemokines for neutrophil activation. *Blood* 1994; 84: 2329–2339.

66 Piccardoni P, Evangelista V, Piccoli A, de Gaetano G, Walz A, Cerletti C. Thrombin-activated human platelets release two NAP-2 variants that stimulate polymorphonuclear leukocytes. *Thromb Haemost* 1996; 76: 780–785.

67 Yan Z, Holt JC, Stewart GJ, Niewiarowski S. Neutrophil-activating intercrine secreted by porcine platelets is active without proteolytic processing. *Am J Physiol* 1993; 265: C1396–C1404.

68 Schwartz BS. Monocyte synthesis of thrombospondin. The role of platelets. *J Biol Chem* 1989; 264: 7512–7517.

69 Scheuerer B, Ernst M, Durrbaum-Landmann I et al. The CXC-chemokine platelet factor 4 promotes monocyte survival and induces monocyte differentiation into macrophages. *Blood* 2000; 95: 1158–1166.

70 Curtiss LK, Black AS, Takagi Y, Plow EF. New mechanism for foam cell generation in atherosclerotic lesions. *J Clin Invest* 1987; 80: 367–373.

71 Forlow SB, McEver RP, Nollert MU. Leukocyte–leukocyte interactions mediated by platelet microparticles under flow. *Blood* 2000; 95: 1317–1323.

Cambridge Books Online © Cambridge University Press, 2009

72 Hernandez R, Alemany M, Bozzo J, Ordinas A, Bastida E. Influence of co-incubation and cell number of platelets and polymorphonuclear leukocytes in cellular inhibition and activation phenomena. *Thromb Res* 1994; 74: 255–263.
73 Moon DG, van der Zee H, Weston LK, Gudewicz PW, Fenton JW, Kaplan JE. Platelet modulation of neutrophil superoxide anion production. *Thromb Haemost* 1990; 63: 91–96.
74 Dallegri F, Ballestrero A, Ottonello L, Patrone F. Platelets as inhibitory cells in neutrophil-mediated cytolysis. *J Lab Clin Med* 1989; 114: 502–509.
75 Michelson AD, Barnard MR, Hechtman HB et al. In vivo tracking of platelets: circulating degranulated platelets rapidly lose surface P-selectin but continue to circulate and function. *Proc Natl Acad Sci USA* 1996; 93: 11877–11882.
76 Gamble JR, Skinner MP, Berndt MC, Vadas MA. Prevention of activated neutrophil adhesion to endothelium by soluble adhesion protein GMP140. *Science* 1990; 249: 414–417.
77 Iwabuchi K, Yamashita T. Platelet-derived neutrophil adherence-inhibiting factor in humans. *Blood* 1990; 76: 2368–2373.
78 Gamble JR, Vadas MA. Endothelial adhesiveness for blood neutrophils is inhibited by transforming growth factor-beta. *Science* 1988; 242: 97–99.
79 Wong CS, Gamble JR, Skinner MP, Lucas CM, Berndt MC, Vadas MA. Adhesion protein GMP140 inhibits superoxide anion release by human neutrophils. *Proc Natl Acad Sci USA* 1991; 88: 2397–2401.
80 Wilson E, Laster SM, Gooding LR, Lambeth JD. Platelet-derived growth factor stimulates phagocytosis and blocks agonist-induced activation of the neutrophil oxidative burst: a possible cellular mechanism to protect against oxygen radical damage. *Proc Natl Acad Sci USA* 1987; 84: 2213–2217.
81 Harter L, Petersen F, Flad HD, Brandt E. Connective tissue-activating peptide III desensitizes chemokine receptors. *J Immunol* 1994; 153: 5698–5708.
82 Ehlert JE, Ludwig A, Grimm TA, Lindner B, Flad HD, Brandt E. Down-regulation of neutrophil functions by the ELR^+ CXC chemokine platelet basic protein. *Blood* 2000; 96: 2965–2972.
83 Semeraro N, Locati D, Colucci M. Leucocyte-dependent platelet activation: an alternative pathway for initiation of blood clotting in inflammation. *Agents Actions* 1981; 11: 553–555.
84 Niemetz J, Marcus AJ. The stimulatory effect of platelets and platelet membranes on the procoagulant activity of leukocytes. *J Clin Invest* 1974; 54: 1437–1443.
85 Nemerson Y. Tissue factor and hemostasis. *Blood* 1988; 71: 1–8.
86 Semeraro N, Biondi A, Lorenzet R, Locati D, Mantovani A, Donati MB. Direct induction of tissue factor synthesis by endotoxin in human macrophages from diverse anatomical sites. *Immunology* 1983; 50: 529–535.
87 Pinder PB, Hunt JA, Zacharski LR. In vitro stimulation of monocyte tissue factor activity by autologous platelets. *Am J Hematol* 1985; 19: 317–325.
88 Osterud B, Bjorklid E. The production and availability of tissue thromboplastin in cellular populations of whole blood exposed to various concentrations of endotoxin. An assay for detection of endotoxin. *Scand J Haematol* 1982; 29: 175–184.
89 Lorenzet R, Niemetz J, Marcus AJ, Broekman MJ. Enhancement of mononuclear procoagulant activity by platelet 12-hydroxyeicosatetraenoic acid. *J Clin Invest* 1986; 78: 418–423.
90 Engstad CS, Lia K, Rekdal O, Olsen JO, Osterud B. A novel biological effect of platelet factor 4 (PF4): enhancement of LPS-induced tissue factor activity in monocytes. *J Leukoc Biol* 1995; 58: 575–581.
91 Palabrica T, Lobb R, Furie BC et al. Leukocyte accumulation promoting fibrin deposition is mediated in vivo by P-selectin on adherent platelets. *Nature* 1992; 359: 848–851.
92 Celi A, Pellegrini G, Lorenzet R et al. P-selectin induces the expression of tissue factor on monocytes. *Proc Natl Acad Sci USA* 1994; 91: 8767–8771.
93 Amirkhosravi A, Alexander M, May K et al. The importance of platelets in the expression of monocyte tissue factor antigen measured by a new whole blood flow cytometric assay. *Thromb Haemost* 1996; 75: 87–95.
94 Halvorsen H, Olsen JO, Osterud B. Granulocytes enhance LPS-induced tissue factor activity in monocytes via an interaction with platelets. *J Leukoc Biol* 1993; 54: 275–282.
95 Pellegrini G, Malandra R, Celi A, Furie BC, Furie B, Lorenzet R. 12-Hydroxyeicosatetraenoic acid upregulates P-selectin-induced tissue factor activity on monocytes. *FEBS Lett* 1998; 441: 463–466.
96 André P, Hartwell D, Hrachovinova I, Saffaripour S, Wagner DD. Pro-coagulant state resulting from high levels of soluble P-selectin in blood. *Proc Natl Acad Sci USA* 2001; 97: 13835–13840.
97 Rauch U, Bonderman D, Bohrmann B et al. Transfer of tissue factor from leukocytes to platelets is mediated by CD15 and tissue factor. *Blood* 2001; 96: 170–175.
98 Marcus AJ, Broekman MJ, Safier LB, Ullman HL, Islam N. Formation of leukotrienes and other hydroxy acids during platelet-neutrophil interactions in vitro. *Biochem Biophys Res Commun* 1982; 109: 130–137.
99 Gresele P, Arnout J, Coene MC, Deckmyn H, Vermylen J. Leukotriene B4 production by stimulated whole blood: comparative studies with isolated polymorphonuclear cells. *Biochem Biophys Res Commun* 1986; 137: 334–342.
100 Fiore S, Serhan CN. Formation of lipoxins and leukotrienes during receptor-mediated interactions of human platelets and recombinant human granulocyte/macrophage colony-stimulating factor-primed neutrophils. *J Exp Med* 1990; 172: 1451–1457.
101 Palmantier R, Borgeat P. Thrombin-activated platelets promote leukotriene B4 synthesis in polymorphonuclear leucocytes stimulated by physiological agonists. *Br J Pharmacol* 1991; 103: 1909–1916.
102 Maclouf JA, Murphy RC. Transcellular metabolism of neutrophil-derived leukotriene A4 by human platelets. A potential cellular source of leukotriene C4. *J Biol Chem* 1988; 263: 174–181.

Cambridge Books Online © Cambridge University Press, 2009

103 Serhan CN, Sheppard K-A. Lipoxin formation during human neutrophil-platelet interactions. Evidence for the transformation of leukotriene A_4 by platelet 12-lipoxygenase in vitro. *J Clin Invest* 1990; 85: 772–780.
104 Maugeri N, Evangelista V, Piccardoni P et al. Transcellular metabolism of arachidonic acid: increased platelet thromboxane generation in the presence of activated polymorphonuclear leukocytes. *Blood* 1992; 80: 447–451.
105 McCulloch RK, Croft KD, Vandongen R. Enhancement of platelet 12-HETE production in the presence of polymorphonuclear leukocytes during calcium ionophore stimulation. *Biochim Biophys Acta* 1992; 1133: 142–146.
106 Palmantier R, Borgeat P. Transcellular metabolism of arachidonic acid in platelets and polymorphonuclear leukocytes activated by physiological agonists: enhancement of leukotriene B_4 synthesis. In: *Cell–cell interactions in the release of inflammatory mediators*. Wong PY-K, Serhan CN, eds. Plenum Press, New York,1991; pp 73–89.
107 Brady HR, Serhan CN. Adhesion promotes transcellular leukotriene biosynthesis during neutrophil-glomerular endothelial cell interactions: inhibition by antibodies against CD18 and L-selectin. *Biochem Biophys Res Commun* 1992; 186: 1307–1314.
108 Jungi TW, Spycher MO, Nydegger UE, Barandun S. Platelet–leukocyte interaction: selective binding of thrombin-stimulated platelets to human monocytes, polymorphonuclear leukocytes, and related cell lines. *Blood* 1986; 67: 629–636.
109 Larsen E, Celi A, Gilbert GE et al. PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. *Cell* 1989; 59: 305–312.
110 Hamburger SA, McEver RP. GMP-140 mediates adhesion of stimulated platelets to neutrophils. *Blood* 1990; 75: 550–554.
111 Furie B, Furie BC. The molecular basis of platelet and endothelial cell interaction with neutrophils and monocytes: role of P-selectin and the P-selectin ligand, PSGL-1. *Thromb Haemost* 1995; 74: 224–227.
112 McEver RP, Cummings RD. Role of PSGL-1 binding to selectins in leukocyte recruitment. *J Clin Invest* 1997; 100: 485–492.
113 Varki A. Selectin ligands: will the real ones please stand up? *J Clin Invest* 1997; 99: 158–162.
114 Yang J, Furie BC, Furie B. The biology of P-selectin glycoprotein ligand-1: its role as a selectin counterreceptor in leukocyte–endothelial and leukocyte–platelet interaction. *Thromb Haemost* 1999; 81: 1–7.
115 Lawrence MB, Springer TA. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell* 1991; 65: 859–873.
116 Alon R, Hammer DA, Springer TA. Lifetime of the P-selectin-carbohydrate bond and its response to tensile force in hydrodynamic flow. *Nature* 1995; 374: 539–542.
117 Mehta P, Cummings RD, McEver RP. Affinity and kinetic analysis of P-selectin binding to P-selectin glycoprotein ligand-1. *J Biol Chem* 1998; 273: 32506–32513.
118 Schmidtke DW, Diamond SL. Direct observation of membrane tethers formed during neutrophil attachment to platelets or P-selectin under physiological flow. *J Cell Biol* 2000; 149: 719–729.
119 Bullard DC, Kunkel EJ, Kubo H et al. Infectious susceptibility and severe deficiency of leukocyte rolling and recruitment in E-selectin and P-selectin double mutant mice. *J Exp Med* 1996; 183: 2329–2336.
120 Frenette PS, Mayadas TN, Rayburn H, Hynes RO, Wagner DD. Susceptibility to infection and altered hematopoiesis in mice deficient in both P- and E-selectins. *Cell* 1996; 84: 563–574.
121 Buttrum SM, Hatton R, Nash GB. Selectin-mediated rolling of neutrophils on immobilized platelets. *Blood* 1993; 82: 1165–1174.
122 Sheikh S, Nash GB. Continuous activation and deactivation of integrin CD11b/CD18 during de novo expression enables rolling neutrophils to immobilize on platelets. *Blood* 1996; 87: 5040–5050.
123 Kuijper PHM, Gallardo Torres HI, van der Linden JAM et al. Platelet-dependent primary hemostasis promotes selectin- and integrin-mediated neutrophil adhesion to damaged endothelium under flow conditions. *Blood* 1996; 87: 3271–3281.
124 Yeo EL, Sheppard J-AI, Feuerstein IA. Role of P-selectin and leukocyte activation in polymorphonuclear cell adhesion to surface adherent activated platelets under physiologic shear conditions (an injury vessel wall model). *Blood* 1994; 83: 2498–2507.
125 Evangelista V, Manarini S, Rotondo S et al. Platelet/polymorphonuclear leukocyte interaction in dynamic conditions: evidence of adhesion cascade and cross talk between P-selectin and the $\beta 2$ integrin CD11b/CD18. *Blood* 1996; 88: 4183–4194.
126 Cabanas C, Hogg N. Ligand intercellular adhesion molecule 1 has a necessary role in activation of integrin lymphocyte function-associated molecule 1. *Proc Natl Acad Sci USA* 1993; 90: 5838–5842.
127 Konstantopoulos K, Neelamegham S, Burns AR et al. Venous levels of shear support neutrophil-platelet adhesion and neutrophil aggregation via P-selectin and $\beta 2$-integrin. *Circulation* 1998; 98: 873–882.
128 Diacovo TG, Roth SJ, Buccola JM, Bainton DF, Springer TA. Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the $\beta 2$-integrin CD11b/CD18. *Blood* 1996; 88: 146–157.
129 Diacovo TG, deFougerolles AR, Bainton DF, Springer TA. A functional integrin ligand on the surface of platelets: intercellular adhesion molecule-2. *J Clin Invest* 1994; 94: 1243–1251.
130 Weber C, Springer TA. Neutrophil accumulation on activated, surface-adherent platelets in flow is mediated by interaction of Mac-1 with fibrinogen bound to αIIbβ3 and stimulated by platelet-activating factor. *J Clin Invest* 1997; 100: 2085–2093.
131 Altieri DC, Plescia J, Plow EF. The structural motif glycine 190-valine 202 of the fibrinogen γ chain interacts with CD11b/CD18 integrin (α_M β_2, Mac-1) and promotes leukocyte adhesion. *J Biol Chem* 1993; 268: 1847–1853.

Cambridge Books Online © Cambridge University Press, 2009

132 Spangenberg P, Redlich H, Bergmann I, Lösche W, Götzrath M, Kehrel B. The platelet glycoprotein IIb/IIIa complex is involved in the adhesion of activated platelets to leukocytes. *Thromb Haemost* 1993; 70: 514–521.

133 Simon DI, Chen Z, Xu H et al. Platelet glycoprotein Ibalpha is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *J Exp Med* 2000; 192: 193–204.

134 Diamond MS, Springer TA. The dynamic regulation of integrin adhesiveness. *Curr Biol* 1994; 4: 506–517.

135 Zimmerman GA, McIntyre TM, Prescott SM. Adhesion and signaling in vascular cell-cell interactions. *J Clin Invest* 1996; 98: 1699–1702.

136 Lorant DE, Patel KD, McIntyre TM, McEver RP, Prescott SM, Zimmerman GA. Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: a juxtacrine system for adhesion and activation of neutrophils. *J Cell Biol* 1991; 115: 223–234.

137 Cooper D, Butcher CM, Berndt MC, Vadas MA. P-selectin interacts with a β_2-integrin to enhance phagocytosis. *J Immunol* 1994; 153: 3199–3209.

138 Evangelista V, Manarini S, Sideri R et al. Platelet/polymorphonuclear leukocyte interaction: P-selectin triggers protein-tyrosine phosphorylation-dependent CD11b/CD18 adhesion: role of PSGL-1 as a signaling molecule. *Blood* 1999; 93: 876–885.

139 Blanks JE, Moll T, Eytner R, Vestweber D. Stimulation of P-selectin glycoprotein ligand-1 on mouse neutrophils activates β2-integrin mediated cell attachment to ICAM-1. *Eur J Immunol* 1998; 28: 433–443.

140 Hidari KI, Weyrich AS, Zimmerman GA, McEver RP. Engagement of P-selectin glycoprotein ligand-1 enhances tyrosine phosphorylation and activates mitogen-activated protein kinases in human neutrophils. *J Biol Chem* 1997; 272: 28750–28756.

141 Piccardoni P, Sideri R, Manarini S et al. Platelet/polymorphonuclear leukocyte adhesion: a new role for SRC kinases in Mac-1 adhesive function triggered by P-selectin. *Blood* 2001; 98: 108–116.

142 Merhi Y, Provost P, Guidoin R, Latour JG. Importance of platelets in neutrophil adhesion and vasoconstriction after deep carotid arterial injury by angioplasty in pigs. *Arterioscler Thromb Vasc Biol* 1997; 17: 1185–1191.

143 Bednar M, Smith B, Pinto A, Mullane KM. Neutrophil depletion suppresses in-labeled platelet accumulation in infarcted myocardium. *J Cardiovasc Pharmacol* 1985; 7: 906–912.

144 Romson JL, Hook BG, Kunkel SL, Abrams GD, Schork MA, Lucchesi BR. Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. *Circulation* 1983; 67: 1016–1023.

145 Mullane KM, Read N, Salmon JA, Moncada S. Role of leukocytes in acute myocardial infarction in anesthetized dogs: relationship to myocardial salvage by anti-inflammatory drugs. *J Pharmacol Exp Ther* 1984; 228: 510–522.

146 Simpson PJ, Todd III RF, Fantone JC, Mickelson JK, Griffin JD, Lucchesi BR. Reduction of experimental canine myocardial reperfusion injury by a monoclonal antibody (anti-Mo1, anti-CD11b) that inhibits leukocyte adhesion. *J Clin Invest* 1988; 81: 624–629.

147 Connell RS, Swank RL, Webb MC. The development of pulmonary ultrastructural lesions during hemorrhagic shock. *J Trauma* 1975; 15: 116–129.

148 Stimler NP, Hugli TE, Bloor CM. Pulmonary injury induced by C3a and C5a anaphilotoxins. *Am J Pathol* 1980; 100: 327–348.

149 Tvedten HV, Till GO, Ward PA. Mediators of lung injury in mice following systemic activation of complement. *Lab Invest* 1985; 119: 92–100.

150 Issekutz AC, Ripley M, Jackson JR. Role of neutrophils in the deposition of platelets during acute inflammation. *Lab Invest* 1983; 49: 716–724.

151 Johnson RJ, Alpers CE, Pritzl P et al. Platelets mediate neutrophil-dependent immune complex nephritis in the rat. *J Clin Invest* 1988; 82: 1225–1235.

152 Lehr H-A, Olofsson AM, Carew TE et al. P-selectin mediates the interaction of circulating leukocytes with platelets and microvascular endothelium in response to oxidized lipoprotein in vivo. *Lab Invest* 1994; 71: 380–386.

153 Hagberg IA, Roald HE, Lyberg T. Adhesion of leukocytes to growing arterial thrombi. *Thromb Haemost* 1998; 80: 852–858.

154 Wyncoll DL, Evans TW. Acute respiratory distress syndrome. *Lancet* 1999; 354: 497–501.

155 Lefer AM, Campbell B, Scalia R, Lefer DJ. Synergism between platelets and neutrophils in provoking cardiac dysfunction after ischemia and reperfusion: role of selectins. *Circulation* 1998; 98: 1322–1328.

156 Merhi Y, Lacoste L-L, Lam JYT. Neutrophil implications in platelet deposition and vasoconstriction after deep arterial injury by angioplasty in pigs. *Circulation* 1994; 90: 997–1002.

157 Sala A, Aliev GM, Rossoni G et al. Morphological and functional changes of coronary vasculature caused by transcellular biosynthesis of sulfidopeptide leukotrienes in isolated heart of rabbit. *Blood* 1996; 87: 1824–1832.

158 Rossoni G, Sala A, Berti F et al. Myocardial protection by the leukotriene synthesis inhibitor BAY X1005. *J Pharmacol Exp Ther* 1996; 276: 335–341.

159 Sala A, Rossoni G, Berti F et al. Monoclonal anti-CD18 antibody prevents transcellular biosynthesis of cysteinyl leukotrienes in vitro and in vivo and protects against leukotriene-dependent increase in coronary vascular resistance and myocardial stiffness. *Circulation* 2000; 101: 1436–1440.

160 Evangelista V, Celardo A, Dell'Elba G et al. Platelet contribution to leukotriene production in inflammation: in vivo evidence in the rabbit. *Thromb Haemost* 1999; 81: 442–448.

161 Celardo A, Dell'Elba G, Manarini S, de Gaetano G, Cerletti C, Evangelista V. Thromboxane A_2 accounts for transient myocardial ischaemia following injection of the inflammatory peptide fMLP in the rabbit. *Haematologica* 2000; 85 Suppl: 8–9.

162 Roque M, Fallon JT, Badimon JJ, Zhang WX, Taubman MB,

Cambridge Books Online © Cambridge University Press, 2009

Reis ED. Mouse model of femoral artery denudation injury associated with the rapid accumulation of adhesion molecules on the luminal surface and recruitment of neutrophils. *Arterioscler Thromb Vasc Biol* 2001; 20: 335–342.

163 Hayashi S, Watanabe N, Nakazawa K et al. Roles of P-selectin in inflammation, neointimal formation, and vascular remodeling in balloon-injured rat carotid arteries. *Circulation* 2001; 102: 1710–1717.

164 Kumar A, Hoover JL, Simmons CA, Lindner V, Shebuski RJ. Remodeling and neointimal formation in the carotid artery of normal and P-selectin-deficient mice. *Circulation* 1997; 96: 4333–4342.

165 Frenette PS, Wagner DD. Insights into selectin function from knockout mice. *Thromb Haemost* 1997; 78: 60–64.

166 Connolly ES, Winfree CJ, Prestigiacomo CJ et al. Exacerbation of cerebral injury in mice that express the P-selectin gene: identification of P-selectin blockade as a new target for the treatment of stroke. *Circ Res* 1997; 81: 304–310.

167 Naka Y, Toda K, Kayano K, Oz MC, Pinsky DJ. Failure to express the P-selectin gene or P-selectin blockade confers early pulmonary protection after lung ischemia or transplantation. *Proc Natl Acad Sci USA* 1997; 94: 757–761.

168 Sawaya DE, Zibari GB, Minardi A et al. P-selectin contributes to the initial recruitment of rolling and adherent leukocytes in hepatic venules after ischemia/reperfusion. *Shock* 1999; 12: 227–232.

169 Singh I, Zibari GB, Brown MF et al. Role of P-selectin expression in hepatic ischemia and reperfusion injury. *Clin Transplant* 1999; 13: 76–82.

170 Palazzo AJ, Jones SP, Anderson DC, Granger DN, Lefer DJ. Coronary endothelial P-selectin in pathogenesis of myocardial ischemia-reperfusion injury. *Am J Physiol* 1998; 275: H1865-H1872.

171 Kanwar S, Smith CW, Kubes P. An absolute requirement for P-selectin in ischemia/reperfusion-induced leukocyte recruitment in cremaster muscle. *Microcirculation* 1998; 5: 281–287.

172 Manka D, Collins RG, Ley K, Beaudet AL, Sarembock IJ. Absence of P-Selectin, but not Intercellular Adhesion Molecule-1, attenuates neointimal growth after arterial injury in Apolipoprotein E-deficient mice. *Circulation* 2001; 103: 1000–1005.

173 Rogers C, Edelman ER, Simon DI. A mAb to the beta2-leukocyte integrin Mac-1 (CD11b/CD18) reduces intimal thickening after angioplasty or stent implantation in rabbits. *Proc Natl Acad Sci USA* 1998; 95: 10134–10139.

174 Simon DI, Dhen Z, Seifert P, Edelman ER, Ballantyne CM, Rogers C. Decreased neointimal formation in Mac-1(−/−) mice reveals a role for inflammation in vascular repair after angioplasty. *J Clin Invest* 2000; 105: 293–300.

175 Smyth SS, Reis ED, Zhang W, Fallon JT, Gordon RE, Coller BS. β3-integrin-deficient mice but not P-selectin-deficient mice develop intimal hyperplasia after vascular injury: correlation with leukocyte recruitment to adherent platelets 1 hour after injury. *Circulation* 2001; 103: 2501–2507.

176 Welt FG, Edelman ER, Simon DI, Rogers C. Neutrophil, not macrophage, infiltration precedes neointimal thickening in balloon-injured arteries. *Arterioscler Thromb Vasc Biol* 2001; 20: 2553–2558.

177 Ernst E, Hammerschmidt DE, Bagge U, Matrai A, Dormandy JA. Leukocytes and the risk of ischemic diseases. *JAMA* 1987; 257: 2318–2324.

178 Danesh J, Collins R, Appleby P, Peto R. Association of fibrinogen, C-reactive protein, albumin, or leukocyte count with coronary heart disease: meta-analyses of prospective studies. *JAMA* 1998; 279: 1477–1482.

179 Danesh J, Whincup P, Walker M et al. Low grade inflammation and coronary heart disease: prospective study and updated meta-analyses. *BMJ* 2001; 321: 199–204.

180 Lowe GDO, Machado SG, Krol WF, Barton BA, Forbes CD. White blood cell count and haematocrit as predictors of coronary recurrence after myocardial infarction. *Thromb Haemost* 1985; 54: 700–703.

181 Cortellaro M, Boschetti C, Cofrancesco E et al. The PLAT Study: hemostatic function in relation to atherothrombotic ischemic events in vascular disease patients. Principal results. Progetto Lombardo Atero-Trombosi (PLAT) Study Group. *Arterioscler Thromb* 1992; 12: 1063–1070.

182 Mehta J, Dinerman J, Mehta P et al. Neutrophil function in ischemic heart disease. *Circulation* 1989; 79: 549–556.

183 Mazzone A, De Servi S, Ricevuti G et al. Increased expression of neutrophil and monocyte adhesion molecules in unstable coronary artery disease. *Circulation* 1993; 88: 358–363.

184 Cannon CP, McCabe CH, Wilcox RG, Bentley JH, Braunwald E. Association of white blood cell count with increased mortality in acute myocardial infarction and unstable angina pectoris. *Am J Cardiol* 2001; 87: 636–639.

185 Lip GY, Blann AD, Zarifis J, Beevers M, Lip PL, Beevers DG. Soluble adhesion molecule P-selectin and endothelial dysfunction in essential hypertension: implications for atherogenesis? A preliminary report. *J Hypertens* 1995; 13: 1674–1678.

186 Davì G, Romano M, Mezzetti A et al. Increased levels of soluble P-selectin in hypercholesterolemic patients. *Circulation* 1998; 97: 953–957.

187 Blann AD, Dobrotova M, Kubisz P, McCollum CN. von Willebrand factor, soluble P-selectin, tissue plasminogen activator. *Thromb Haemost* 1995; 74: 626–630.

188 Ikeda H, Takajo Y, Ichiki K et al. Increased soluble form of P-selectin in patients with unstable angina. *Circulation* 1995; 92: 1693–1696.

189 Sakurai S, Inoue A, Koh CS, Owa M, Yanagisawa N. Soluble form of selectins in blood of patients with acute myocardial infarction and coronary intervention. *Vasc Med* 1997; 2: 163–168.

190 Shimomura H, Ogawa H, Arai H et al. Serial changes in plasma levels of soluble P-selectin in patients with acute myocardial infarction. *Am J Cardiol* 1998; 81: 397–400.

191 Ridker PM, Buring JE, Rifai N. Soluble P-selectin and the risk of future cardiovascular events. *Circulation* 2001; 103: 491–495.

Cambridge Books Online © Cambridge University Press, 2009

192 Ott I, Neumann F-J, Gawaz M, Schmitt M, Schömig A. Increased neutrophil–platelet adhesion in patients with unstable angina. *Circulation* 1996; 94: 1239–1246.

193 Mickelson JK, Lakkis NM, Villarreal-Levy G, Hughes BJ, Smith CW. Leukocyte activation with platelet adhesion after coronary angioplasty: a mechanism for recurrent disease? *J Am Coll Cardiol* 1996; 28: 345–353.

194 Furman MI, Benoit SE, Barnard MR et al. Increased platelet reactivity and circulating monocyte–platelet aggregates in patients with stable coronary artery disease. *J Am Coll Cardiol* 1998; 31: 352–358.

195 Kassirer M, Zeltser D, Prochorov V et al. Increased expression of the CD11b/CD18 antigen on the surface of peripheral white blood cells in patients with ischemic heart disease: further evidence for smoldering inflammation in patients with atherosclerosis. *Am Heart J* 1999; 138: 555–559.

196 Meisel SR, Shapiro H, Radnay J et al. Increased expression of neutrophil and monocyte adhesion molecules LFA-1 and Mac-1 and their ligand ICAM-1 and VLA-4 throughout the acute phase of myocardial infarction: possible implications for leukocyte aggregation and microvascular plugging. *J Am Coll Cardiol* 1998; 31: 120–125.

197 Mickelson JK, Ali MN, Kleiman NS et al. Chimeric 7E3 Fab (ReoPro) decreases detectable CD11b on neutrophils from patients undergoing coronary angioplasty. *J Am Coll Cardiol* 1999; 33: 97–106.

198 De Servi S, Mazzone A, Ricevuti G et al. Clinical and angiographic correlates of leukocyte activation in unstable angina. *J Am Coll Cardiol* 1995; 26: 1146–1150.

199 Neumann FJ, Zohlnhofer D, Fakhoury L, Ott I, Gawaz M, Schomig A. Effect of glycoprotein IIb/IIIa receptor blockade on platelet–leukocyte interaction and surface expression of the leukocyte integrin Mac-1 in acute myocardial infarction. *J Am Coll Cardiol* 1999; 34: 1420–1426.

200 De Servi S, Mazzone A, Ricevuti G et al. Expression of neutrophil and monocyte CD11B/CD18 adhesion molecules at different sites of the coronary tree in unstable angina pectoris. *Am J Cardiol* 1996; 78: 564–568.

201 Neumann FJ, Marx N, Gawaz M et al. Induction of cytokine expression in leukocytes by binding of thrombin-stimulated platelets. *Circulation* 1997; 95: 2387–2394.

202 Renesto P, Ferrer-Lopez P, Chignard M. Interference of recombinant eglin C, a proteinase inhibitor extracted from leeches, with neutrophil-mediated platelet activation. *Lab Invest* 1990; 62: 409–416.

203 Evangelista V, Piccardoni P, Maugeri N, de Gaetano G, Cerletti C. Inhibition by heparin of platelet activation induced by neutrophil-derived cathepsin G. *Eur J Pharmacol* 1992; 216: 401–405.

204 Renesto P, Balloy V, Kamimura T, Masuda K, Imaizumi A, Chignard M. Inhibition by recombinant SLPI and half-SLPI (Asn^{55}-Ala^{107}) of elastase and cathepsin G activities: consequence for neutrophil-platelet cooperation. *Br J Pharmacol* 1993; 108: 1100–1106.

205 Evangelista V, Piccardoni P, White JG, de Gaetano G, Cerletti C. Cathepsin G-dependent platelet stimulation by activated polymorphonuclear leukocytes and its inhibition by antiproteinases: role of P-selectin-mediated cell-cell adhesion. *Blood* 1993; 81: 2947–2957.

206 Skinner MP, Lucas CM, Burns GF, Chesterman CN, Berndt MC. GMP-140 binding to neutrophils is inhibited by sulfated glycans. *J Biol Chem* 1991; 266: 5371–5374.

207 Ferrer-Lopez P, Renesto P, Prevost MC, Gounon P, Chignard M. Heparins inhibit neutrophil-induced platelet activation via cathepsin G. *J Lab Clin Med* 1992; 119: 231–239.

208 Bazzoni G, Beltrán-Nuñez A, Mascellani G, Bianchini P, Dejana E, Del Maschio A. Effect of heparin, dermatan sulfate, and related oligo-derivates on human polymorphonuclear leukocyte functions. *J Lab Clin Med* 1993; 121: 268–275.

209 Diamond MS, Alon R, Parkos CA, Quinn MT, Springer TA. Heparin is an adhesive ligand for the leukocyte integrin Mac-1 (CD11b/CD1). *J Cell Biol* 1995; 130: 1473–1482.

210 Peter K, Schwarz M, Conradt C et al. Heparin inhibits ligand binding to the leukocyte integrin Mac-1 (CD11b/CD18). *Circulation* 1999; 100: 1533–1539.

211 Lever R, Hoult JR, Page CP. The effects of heparin and related molecules upon the adhesion of human polymorphonuclear leucocytes to vascular endothelium in vitro. *Br J Pharmacol* 2000; 129: 533–540.

212 Rajtar G, Marchi E, de Gaetano G, Cerletti C. Effects of glycosaminoglycans on platelet and leucocyte function: role of N-sulfation. *Biochem Pharmacol* 1993; 46: 958–960.

213 Evangelista V, Piccardoni P, de Gaetano G, Cerletti C. Defibrotide inhibits platelet activation by cathepsin G released from stimulated polymorphonuclear leukocytes. *Thromb Haemost* 1992; 67: 660–664.

214 Colli S, Tremoli E. Multiple effects of dipyridamole on neutrophils and mononuclear leukocytes: adenosine-dependent and adenosine-independent mechanisms. *J Lab Clin Med* 1991; 118: 136–145.

215 European Working Group on Critical Leg Ischaemia. Second European consensus document on chronic critical leg ischemia. *Circulation* 1991; 84 Suppl: IV-1–IV-26.

216 Dinermann JL, Mehta JL, Salden TGP. Increased neutrophil elastase release in unstable angina pectoris and acute myocardial infarction. *J Am Coll Cardiol* 1990; 15: 1559–1563.

217 Kupatt C, Habazettl H, Hanusch P et al. c7E3Fab reduces postischemic leukocyte-thrombocyte interaction mediated by fibrinogen: implications for myocardial reperfusion injury. *Arterioscler Thromb Vasc Biol* 2000; 20: 2226–2232.

218 Fredrickson BJ, Turner NA, Kleiman NS et al. Effects of abciximab, ticlopidine, and combined Abciximab/Ticlopidine therapy on platelet and leukocyte function in patients undergoing coronary angioplasty. *Circulation* 2000; 101: 1122–1129.

219 Toombs CF, DeGraaf GL, Martin JP, Geng JG, Anderson DC, Shebuski RJ. Pretreatment with a blocking monoclonal antibody to P-selectin accelerates pharmacological thrombolysis in a primate model of arterial thrombosis. *J Pharmacol Exp Ther* 1995; 275: 941–949.

220 Kumar A, Villani MP, Patel UK, Keith JC, Schaub RG. Recombinant soluble form of PSGL-1 accelerates thromboly-

Cambridge Books Online © Cambridge University Press, 2009

sis and prevents reocclusion in a porcine model. *Circulation* 1999; 99: 1363–1368.
221 Merhi Y, Provost P, Chauvet P, Theoret JF, Phillips ML, Latour JG. Selectin blockade reduces neutrophil interaction with platelets at the site of deep arterial injury by angioplasty in pigs. *Arterioscler Thromb Vasc Biol* 1999; 19: 372–379.
222 Ueyama T, Ikeda H, Haramaki N, Kuwano K, Imaizumi T. Effects of monoclonal antibody to P-selectin and analogue of sialyl Lewis X on cyclic flow variations in stenosed and endothelium-injured canine coronary arteries. *Circulation* 1997; 95: 1554–1559.
223 Bienvenu JG, Tanguay JF, Theoret JF, Kumar A, Schaub RG, Merhi Y. Recombinant soluble P-Selectin Glycoprotein Ligand-1–Ig reduces restenosis through inhibition of platelet-neutrophil adhesion after double angioplasty in swine. *Circulation* 2001; 103: 1128–1134.
224 Rotondo S, de Gaetano G. Protection from cardiovascular disease by wine and its derived products. Epidemiological evidence and biological mechanisms. *World Rev Nutr Diet* 2000; 87: 90–113.
225 Renaud S, De Lorgeril M. Wine, alcohol, platelets, and the French paradox for coronary heart diseases. *Lancet* 1992; 339: 1523–1526.
226 Rotondo S, Di Castelnuovo A, de Gaetano G. The relationship between wine consumption and cardiovascular risk: from epidemiological evidence to biological plausibility. *Ital Heart J* 2001; 2: 1–8.
227 Rotondo S, Rajtar G, Manarini S et al. Effect of *trans*-resveratrol, a natural polyphenolic compound, on human polymorphonuclear leukocyte function. *Br J Pharmacol* 1998; 123: 1691–1699.
228 Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med* 1999; 340: 115–126.
229 de Gaetano G, Evangelista V, Cerletti C. Should cardiologists forget about platelets and take an interest in blood leukocytes? *Ital Heart J* 2000; 1: 453–456.
230 Frenette PS, Wagner DD. Adhesion molecules – Part 1. *N Engl J Med* 1996; 334: 1526–1529.
231 Bevilacqua MP, Nelson RM, Mannori G, Cecconi O. Endothelial-leukocyte adhesion molecules in human disease. *Annu Rev Med* 1994; 45: 361–378.
232 Ginsberg MH, Ruggeri ZM, Varki AP (series eds). Prospective series: cell adhesion in vascular biology. *J Clin Invest* 1997; 100, Suppl 11: S1–S103.

Cambridge Books Online © Cambridge University Press, 2009

29

Vascular control of platelet function

Scott Willoughby[1] and Joseph Loscalzo[2]

[1]*Cardiology Unit, The Queen Elizabeth Hospital, Woodville, South Australia*
[2]*Whitaker Cardiovascular Institute and Evans Department of Medicine, Boston University School of Medicine, Massachusetts, USA*

Introduction

The vascular endothelium is a dynamic cellular interface between the blood and subendothelial tissue and is critical for the maintenance of blood flow and hemostasis. The endothelium releases substances (Fig. 29.1) that play an important role in the modulation of vascular tone and permeability, as well as blood fluidity. These substances affect vascular tone by causing both vasodilation [prostaglandin I_2 (PGI_2), nitric oxide (NO), and endothelium-derived hyperpolarizing factor (EDHF)] and vasoconstriction [thromboxane A_2 (TXA_2) and endothelin (Et-1)], with the interaction between these two processes ultimately determining the tone of the vessel. In addition to modulating vascular tone, substances released from the endothelium affect platelet adhesion and aggregation. Nitric oxide inhibits platelets adhesion and aggregation, and PGI_2 inhibits platelet aggregation.

Abnormal endothelial responses (endothelial dysfunction) or frank damage to or denudation of the endothelium promotes a prothrombotic state by reducing antiplatelet factors and by inducing adhesion molecule expression. Furthermore, endothelial dysfunction may be involved in the pathogenesis of several cardiovascular disorders, including atherosclerosis, diabetes mellitus, essential hypertension, hypercholesterolemia, and hyperhomocysteinemia.

In this chapter we will review the control of normal endothelial function, with specific emphasis on antiplatelet effects of substances released from the endothelial cell. We will also review the consequences of endothelial dysfunction, giving specific examples in several disease states.

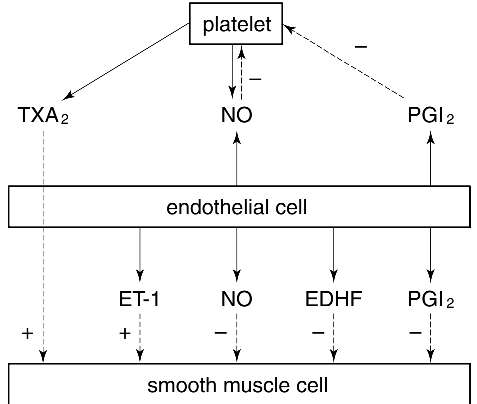


Fig. 29.1. Effect of substances released (solid line) from the endothelial cell. Nitric oxide, PGI_2, and EDHF released from the endothelial cell act on the smooth muscle cell to produce vasodilation (−) of the vessel. In addition, NO and PGI_2 also inhibit platelet activation and aggregation (−). Endothelin-1 released by the endothelial cell and TXA_2 released by platelets produce vasoconstriction (+) of the vessel.

Molecular determinants of endothelial function

Vasoactive substances

Prostaglandin I_2

Prostaglandin I_2, or prostacyclin, is a major product of arachidonic acid metabolism. When hydrolyzed from membrane phospholipids, arachidonic acid is metabolized by cyclooxygenase to the endoperoxide, prostaglandin H_2 (PGH_2). Prostaglandin H_2 is a substrate for a series of enzymes that form PGI_2, PGE_2, and $PGF_{2\alpha}$ as the major prostanoid products in vascular endothelial and smooth muscle cells; however, PGI_2 is produced principally by the endothelial cell[1]. The endothelial production of PGI_2 and

PGE_2 is increased by agonists, such as adenosine 5-diphosphate (ADP)/ adenosine 5-triphosphate (ATP), platelet activating factor (PAF), histamine, leukotrienes C_4 and D_4, thrombin, and bradykinin[2–8].

Prostaglandin I_2 is a potent endothelium-derived vasodilator[9,10] the release of which can be enhanced by hemodynamic forces, such as shear stress and pulsatile pressure, as well as by biochemical mediators, including thrombin, bradykinin, angiotensin II, histamine, platelet-derived growth factor (PDGF), interleukin-1 (IL-1), TXA_2, and adenine nucleotides[11–16]. Prostaglandin I_2 acts by binding to a G protein-coupled receptor on the vascular smooth muscle cell linked to adenylyl cyclase, causing an increase in the intracellular levels of cyclic 3′,5′-adenosine monophosphate (cAMP)[17]. In turn, cyclic AMP activates protein kinase A, which influences calcium regulatory mechanisms, resulting in decreased intracellular calcium concentration, decreased calcium-dependent actomyosin ATPase activity, relaxation of vascular smooth muscle cells, and vasodilation[18].

In addition to its effect on the vascular smooth muscle cell, endothelium-derived PGI_2 also inhibits the activation and aggregation of platelets and promotes their disaggregation[19–21]. This action on platelets is mediated, as well, by increases in intracellular cyclic AMP levels[1,22]. However, unlike NO, which inhibits both adhesion and aggregation of activated platelets partly via a cyclic 3,5-guanosine monophosphate (cyclic GMP)-dependent mechanism, PGI_2 only inhibits platelet activation and aggregation and has no effect on adhesion[23].

The other prostaglandins produced by the metabolism of arachidonic acid may achieve high enough concentrations locally (adjacent to the endothelium) to contribute to vascular homeostasis. Prostaglandin H_2 is a potent vasoconstrictor, indicating that vasodilation by arachidonic acid products, such as PGI_2, depends largely on the conversion of PGH_2 to PGI_2[24]. In a variety of vascular diseases, the conversion of PGH_2 to PGI_2 may be impaired, leading to endothelium-dependent contraction by the precursor prostaglandin endoperoxides[25]. Thus, the conversion of PGH_2 to PGI_2 in endothelial cells may be a key event on which rests the balance between endothelium-dependent vasodilation and constriction, particularly that associated with vascular diseases[24,26].

Nitric oxide

The importance of endothelial NO was first observed in 1980 by Furchgott and Zawadzki, who demonstrated that exposure of an isolated rabbit aorta to acetylcholine induces release of a vasodilator substance[27]. This substance was subsequently termed endothelium-derived relaxing factor (EDRF). Endothelium-derived relaxing factor has since been identified as NO or a closely related redox form of NO[28–32]. Nitric oxide is derived from L-arginine (Fig. 29.2) via NO synthase (NOS) by oxidation of its terminal guanidino nitrogen[33,34]. Molecular oxygen and NADPH are cosubstrates in this reaction[35,36]. There are several isoforms of NOS, one is a constitutive enzyme class that is present in the vascular endothelium (eNOS or NosIII), neuronal cells (nNOS or NosI), and other cell types, while the other is an inducible form of the enzyme (iNOS or Nos II), which has been found in many cell types, including macrophages, neutrophils, and vascular smooth muscle cells[37]. Nitric oxide synthases contain both flavin adenine dinucleotide and mononucleotide[38] and require the presence of several redox-active cofactors, including tetrahydrobiopterin, reduced glutathione, and a heme complex[39–42], for optimal activity.

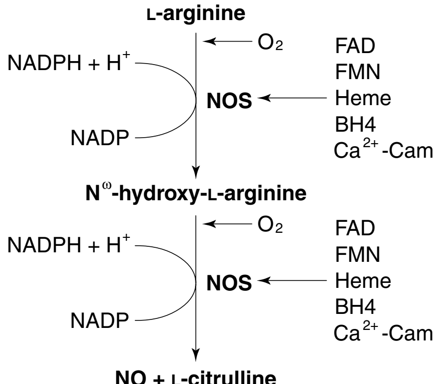


Fig. 29.2. The formation of NO from L-arginine by nitric oxide synthase (NOS). Nitric oxide synthase requires FAD, FMN, Heme, BH_4, and Ca^{2+}-CaM as essential cofactors. *Abbreviations:* BH_4, tetrahydrobiopterin; Ca^{2+}-CaM, calcium-calmodulin; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NADP, nicotinamide adenine dinucleotide phosphate.

In the vascular endothelium, agonists such as acetylcholine and bradykinin stimulate inositol 1,4,5-trisphosphate (IP_3) production by activating the phosphoinositide second messenger system[43]. Inositol 1,4,5-trisphosphate binds to receptors on the endoplasmic reticulum and causes calcium release from intracellular stores; this calcium binds to calmodulin, and, once formed, the calcium/calmodulin complex activates cNOS[44,45]. The rapid and transient production of NO by nNOS allows NO to function in neuronal tissue as a neurotransmitter. Similarly, in the vascular endothelium, eNOS is well suited for its role in maintaining basal vascular tone[46] owing to its capacity to generate low-level NO constitutively.

After production, NO readily diffuses across cell

Cambridge Books Online © Cambridge University Press, 2009

membranes to interact with specific molecular targets[33]. Nitric oxide regulates protein activity by binding reversibly to available acceptor functionalities, including heme iron and thiols[47]. After entering the target cell, NO binds to the heme moiety of soluble guanylyl cyclase and activates the enzyme, which then catalyses the production of cyclic GMP from guanosine 5′-triphosphate (GTP)[48,49]. Cyclic GMP, in turn, serves as a second messenger, activating cyclic GMP-dependent protein kinase, which decreases cytosolic calcium concentration and modulates ion channel functions, leading to relaxation of vascular smooth muscle cells[50]. Nitric oxide can also directly activate calcium-dependent potassium channels in vascular smooth muscle cells, producing hyperpolarization and relaxation[51]. In addition, nitric oxide inhibits platelet aggregation by a guanylyl cyclase mechanism[52,53].

Nitric oxide is continuously produced by the vascular endothelium, and this basal release of NO regulates vascular tone[54,55]. For example, in isolated arteries, the specific NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) produces endothelium-dependent contraction[56], while infusion of L-NMMA in the human forearm induces an increase in peripheral vascular resistance[6,57]. Furthermore, systemic doses of L-NMMA evoke long-lasting increases in blood pressure[58]. Normal NO release opposes the vasoconstrictor responses to clinically relevant stimuli, such as catecholamines[59] and serotonin[60]. Thus, NO synthase inhibition is associated with increased systemic blood pressure, decreased blood flow responses to exercise and local ischemia[61,62], and shortened bleeding time[63]. Inhibition of NO production is also associated with increased adhesion of leukocytes[64] or platelets[65] to cultured endothelial cells. Nitric oxide production by endothelial cells is stimulated by several factors present in the vascular milieu, including acetylcholine, bradykinin, calcium ionophore, adenine nucleotides, thrombin, substance P, neurokinins, vasopressin, oxytocin, uridine triphosphate (UTP), histamine, and $PFG_{2\alpha}$[65–67], although the responsiveness to these agonists exhibits some heterogeneity among vascular cells derived from different species and different vascular beds[66].

Endothelium-derived hyperpolarizing factor

Prostaglandin I_2 and NO are potent endothelial vasodilators; however, not all endothelium-dependent relaxation can be fully explained by their presence alone. For example, endothelium-dependent relaxation and hyperpolarization can be partially or totally resistant to inhibitors of cyclooxygenase and NOS[68–72]. Thus, there is at least one additional endothelial substance, as yet unidentified, that contributes to endothelial hyperpolarization; this has been termed endothelium-derived hyperpolarizing factor (EDHF)[69,73,74].

Although the precise nature of EDHF is not known, in some blood vessels epoxyeicosatrienoic acids, metabolites of P_{450} monooxygenase pathways of arachidonic acid metabolism, have been proposed as possible candidates for EDHF[75–77]. The vasodilator effect of EDHF is mediated by the activation of potassium channels in the vascular smooth muscle cell, with the amplitude of hyperpolarization inversely related to the extracellular concentration of potassium ions[78]. In addition, endothelial relaxation can be blocked by non-selective potassium channel inhibitors[71,79,80]. Some of the actions of EDHF have also been attributed to calcium-dependent potassium channels and potassium ATP channels[81]; however, these responses differ among species[82].

Endothelium-derived hyperpolarizing factor-mediated vasodilation is observed in both humans and animals[83,84]. The contribution of EDHF to endothelium-dependent relaxations increases as vessel size decreases, both in humans and in animals[84,85].

Elevated NO induces an impairment of the EDHF pathway[86,87] through a cyclic GMP-dependent mechanism[86]. This effect of NO appears to involve a reduction in the mobilization of arachidonic acid by agonists[87]. Impairment of EDHF by NO occurs only under conditions of excessive production of NO, such as those obtained in pro-inflammatory states, suggesting a role for the high-flux production of NO by iNOS. In arteries isolated from rats treated chronically with NOS inhibitors, EDHF-mediated relaxation is of the same magnitude as that obtained when NOS is inhibited acutely in vitro[88].

Endothelin

In addition to releasing vasodilatory substances, the endothelium also has the capacity to release/synthesize the vasoconstrictor endothelin (ET). Endothelin acts locally to oppose the vasodilation produced by PGI_2, NO, and EDHF. It is the interactions among these opposing substances that ultimately determine the vasomotor response of the vessel.

Endothelins are divided into three distinct peptide groups, ET-1, ET-2, and ET-3, which are derived from a separate gene product, prepro-endothelin, via 'big' endothelin through a series of subsequent peptide cleavages. Endothelin-1 is an extremely potent vasoconstrictor synthesized by the endothelium that produces long-lasting contractions of vascular smooth muscle. However, vasoconstriction can be prevented by proteases and inhibitors of protein synthesis[89]. Endothelin is synthesized in a two-step process. A pre-propeptide is cleaved by protease to

Cambridge Books Online © Cambridge University Press, 2009

form big ET-1 (39 amino acids), which is further cleaved by the endothelin converting enzyme (ECE) to form the 21-amino acid active peptide[90]. Big ET-1 can be identified intracellularly in endothelial cells, suggesting that ET-1 is synthesized constitutively and not stored. Endothelin-1 is found primarily in the endothelium, whereas ET-2 and ET-3 are present in nonvascular tissue[91]. A variety of stimuli can evoke ET-1 production by the endothelial cell, including IL-1, thrombin, TGF-β, phorbol esters, calcium ionophore, arginine vasopressin, hypoxia, shear stress, and vasoactive substances released by platelets[92–97], although the detection of stimulated levels requires 3 to 6 hours, consistent with alterations in gene expression. Importantly, ET-1 synthesis can also be inhibited by NO[92,98–100].

The endothelin receptor comprises two subtypes: ETa receptors (which have a higher affinity for ET-1 than ET-3) found mainly on the vascular smooth muscle cell and cardiomyocyte[98], and ETb receptors (which are non-selective for ET subtypes) located primarily on the endothelial cell and vascular smooth muscle cell[99]. Thus, ETa and ETb receptors mediate vasoconstriction in a variety of blood vessels and vascular beds, with a large degree of heterogeneity demonstrated among different species[100].

Vasomotion is affected by ETa and ETb receptors located on endothelial and vascular smooth muscle cells[101]. Endothelin-1 acts on ETa and ETb receptors located on vascular smooth muscle cells to elicit long-lasting vasoconstriction. This effect is mediated by the opening of ion (sodium and calcium) channels, which produces an increase in intracellular calcium[102]. In addition, ET-1 can activate ETbi receptors on the endothelium that leads to release of factors, such as NO and PGI_2, which, in turn, lead to an initial vascular smooth muscle relaxation mediated by increased cyclic AMP and cyclic GMP levels, respectively, with subsequent inhibition of L-type calcium channels[103]. Thus, under normal conditions, endothelial ET-1 synthesis contributes to the maintenance of basal vascular tone and blood pressure principally through activation of ETa receptors on vascular smooth muscle cells.

Thromboxane A_2

The endothelial cell metabolizes arachidonic acid via cyclooxygenase, which leads to the secretion of vasoconstrictor substances, such as PGH_2. In addition, thromboxane A_2 is secreted from platelets by the metabolism of arachidonic acid via thromboxane synthase. Prostaglandin H_2 and TXA_2 subsequently act on endoperoxide/thromboxane receptors in vascular smooth muscle cells to induce vasoconstriction and on platelets to induce activation and aggregation[104]. Prostaglandin H_2 has been shown to attenuate endothelium-dependent vasodilation induced by acetylcholine in isolated aortic rings from spontaneously hypertensive rats[105]. However, PGH_2 can also be converted to PGI_2 by the endothelial cell, which then opposes vasoconstriction.

Antiplatelet substances

Prostaglandin I_2

Endothelial cells and platelets can both generate eicosanoids from arachidonic acid via cyclooxygenase. However, the products formed in each cell type have opposing effects. Endothelial cells contain prostacyclin synthetase, which can produce PGI_2 from endoperoxides (PGH_2)[106], while platelets contain thromboxane synthetase, which produces TXA_2[18]. Thromboxane A_2 formation in the platelet induces platelet aggregation, which is accompanied by the platelet release reaction whereby serotonin and other granule components are expelled from platelet stores. Thromboxane A_2 also induces a rise in the concentration of ionized calcium in the platelet cytosol and a decrease in platelet cyclic AMP formation by inhibiting adenylyl cyclase. In addition, TXA_2 acts on vascular smooth muscle cells to produce vasoconstriction. As platelets are unable to resynthesize cyclooxygenase, in the presence of the irreversible cyclooxygenase inhibitor aspirin, TXA_2 production is completely blocked. In contrast, endothelial cells are able to resynthesize cyclooxygenase; thus, aspirin only leads to a transient decrease in PGI_2 production.

Endoperoxides (PGG_2, PGH_2) released from the platelet by the conversion of arachidonic acid can also be used by the endothelium to produce PGI_2[106]. In addition to its potent vasodilatory effects, PGI_2 also inhibits platelet function. Prostaglandin I_2 binds to a specific receptor on the surface of the platelet and stimulates adenylyl cyclase[22]. The resulting increase in platelet cyclic AMP leads to calcium reuptake by the dense tubular system and, thereby, inhibits platelet activation, platelet granule secretion, platelet aggregation, and the development of platelet procoagulant activities[107].

Nitric oxide

Nitric oxide is a potent endothelial product that is principally involved in the regulation of vascular tone and in maintaining the antithrombotic properties of the endothelium. Nitric oxide achieves these ends by decreasing vascular smooth muscle tone and by inhibiting platelet responses, such as platelet adhesion, platelet granule secretion, platelet aggregation, and arachidonic acid liberation[108–110], respectively.

Cambridge Books Online © Cambridge University Press, 2009

Nitric oxide released by the endothelium diffuses readily across the platelet membrane and binds to the heme moiety of soluble guanylyl cyclase. Heme binding leads to guanylyl cyclase activation and an increase in cyclic GMP production, which, in turn, activates a cyclic GMP-dependent protein kinase that phosphorylates myosin light chain kinase and regulates actomyosin ATPase activity[111,112]. Activation of cyclic GMP-dependent protein kinase also leads to phosphorylation of calcium transporters, which decrease intracellular calcium concentration[113]. Nitric oxide also inhibits the normal activation-dependent increase in platelet surface glycoprotein expression, such as P-selectin and the active conformation of glycoprotein (GP) IIb–IIIa[114]. Endothelial NO production is stimulated by shear stress and by substances released during platelet activation (such as ADP and 5-HT), as well as by determinants of the coagulation cascade, such as thrombin[115].

In addition to the effect of endothelial NO, platelets themselves possess both constitutive and, possibly, inducible NOS (cNOS and iNOS), whereby NO is produced during platelet aggregation[116–118]. Platelet aggregation can be enhanced by incubation with inhibitors of NOS and decreased by the NOS substrate L-arginine[119], and this platelet-derived NO pool also limits platelet recruitment to the growing platelet thrombus[116], potentially limiting the self-amplification of platelet thrombus formation in vivo. Thus, NO appears to be involved in the regulation of platelet function and platelet aggregate formation.

The anti-thrombotic properties of the endothelial surface may be a consequence of the combined actions of NO and PGI_2. Prostaglandin I_2 is as potent local vasodilator and a potent inhibitor of platelet aggregation as is NO[120]. By contrast with NO, the physiological antiplatelet effects of PGI_2 are mediated by an increase in cyclic AMP[19]. Nitric oxide also causes vasodilation and inhibits platelet aggregation, but unlike PGI_2, NO also inhibits platelet adhesion to the vascular endothelium[121]. Nitric oxide and PGI_2 have been shown to have synergistic antiaggregatory effects on platelets[108,120], and may act in concert to oppose local vasospasm or thrombus formation at sites where platelets aggregate and the coagulation cascade is activated[115]. It has been proposed that the antiplatelet effects of endothelial NO may prevent thromboembolic events during administration of potent PGI_2 inhibitors, such as aspirin[46].

Phosphoinositide 3-kinase

Phosphoinositide 3-kinase (PI3-kinase) is involved in many cellular functions, including the regulation of glucose transport, mitogenesis, and cytoskeletal rearrangements[122]. Phosphoinositide 3-kinase catalyses the phosphorylation of the inositol ring at the 3′ position of a variety of phosphoinositides[123,124]. In addition, some isoforms can catalyse the phosphorylation of protein serine residues[125,126].

Platelets contain a p85/PI3-kinase[127], which is a heterodimer composed of a p85 regulatory subunit (with two SH2 domains and one SH3 domain) and a catalytic p110 subunit. The p85/PI3-kinase can be activated by G-protein linked receptors, including the thrombin receptor in platelets[128,129]. Recent work in our laboratory has shown that S-nitrosoglutathione, an NO donor, inhibits PI3-kinase activation induced by thrombin receptor-activating peptide (TRAP) in human platelets[122]. In addition, the PI3-kinase inhibitor, wortmannin, when used in conjunction with S-nitrosoglutathione was shown to have additive inhibitory effects on TRAP-induced platelet aggregation. Although wortmannin has previously been shown to mimic the effects of NO on the platelet, such as inhibition of TRAP-induced platelet aggregation[130], the study by Pigazzi and colleagues is the first to demonstrate an inhibitory effect of NO on PI3-kinase in platelets[122].

Phosphoinositide 3-kinase has also been shown to induce NO synthesis when endothelial cells are stimulated by either insulin or vascular endothelial growth factor (VEGF)[131]. For example, endothelial cells pre-treated with wortmannin inhibit the production of NO by insulin[132]. The relative importance of the opposing effects of NO on PI3-kinase activation in platelets and in endothelial cells is yet to be determined.

AKT

The serine/threonine protein kinase AKT (protein kinase B) is a downstream target of PI3-kinase[133,134] and has previously been shown to be a general antiapoptotic effector induced by proangiogenic stimuli[135]. For example, VEGF stimulates AKT, which promotes endothelial cells survival[136,137], and inhibitors of endothelial cell apoptosis, shear stress, and insulin, also activate AKT[138,139]. More recently, AKT has been shown to phosphorylate eNOS directly leading to calcium-independent activation of the enzyme[140,141]. In addition, inhibition of the AKT/PI3-kinase pathway by wortmannin prevents activation of eNOS[141], while activated AKT (delivered via adenovirus-mediated gene transfer) increases basal NO release from endothelial cells[140]. Thus, AKT-dependent phosphorylation of eNOS represents an additional mechanism by which endothelial NO production is modulated. The role of AKT in platelet aggregation and effects of NO on this process are, as yet, unknown.

Cambridge Books Online © Cambridge University Press, 2009

Cyclic GMP-independent mechanisms
Nitric oxide also interacts with target cells by cyclic GMP-independent mechanisms. Nitric oxide can activate cytosolic adenosine 5′-diphosphate (ADP)-ribosyltransferase in human platelets, an enzyme that catalyses the transfer of ADP ribose to glyceraldehyde 3-phosphate dehydrogenase[142]. The addition of ADP ribose to GAPDH inactivates the enzyme and, thereby, slows glycolysis and decreases ATP production[43]. Nitric oxide-mediated injury in processes such as myocardial stunning or neurotoxicity may be secondary to NO stimulation of GAPDH ADP-ribosylation, with the resultant inhibition of glycolysis[43]. There are additional cyclic GMP-independent effects of NO on endothelial cells of possible importance to vascular function. Endothelium-dependent relaxation can occur without a rise in cyclic GMP[51]; this response is attributed to direct activation by NO of calcium-dependent potassium channels[81], which produce relaxation by hyperpolarization of the vascular smooth muscle cell. Furthermore, sodium–potassium ATPase can be activated by NO independent of cyclic GMP[143], which, again, leads to hyperpolarization. Nitric oxide-mediated inhibition of cytosolic calcium influx in platelets[144] activated by strong agonists is a consequence of inhibition of capacitative calcium influx resulting from increased sarcoplasmic/endoplasmic reticulum calcium-ATPase-dependent refilling of intracellular calcium stores[145].

EctoADPase
In addition to inhibiting platelet aggregation via endothelium-derived PGI_2 and NO, the endothelial cell reduces the extent of platelet aggregation by decreasing the local concentration of the platelet agonist ADP via surface ecto-nucleotidase activity. Adenosine 5′-diphosphate released from activated platelets induces platelet recruitment and aggregation by binding to platelet purinergic type-2 receptors (P2Y or P2X)[146]. Adenosine 5′-triphosphate diphosphohydrolase (ADPase, ATPDase, ecto-apyrase, or CD39), located on the luminal surface of the endothelial cell, metabolizes extracellular ATP and ADP to AMP. Within the endothelial cell, AMP is then rapidly metabolized by 5′-nucleotidase to adenosine, which inhibits platelet aggregation[147]. Ecto-ADPase is a glycoprotein that belongs to the E-type ATPase family[148] and hydrolyzes all physiologically occurring nucleoside 5′-triphosphates and 5′-diphosphates equally well[147]. The activity of ecto-ADPDase is dependent on calcium and magnesium, and can be inhibited by divalent cation-chelating agents[149,150].

Ecto-ADPase is insensitive to the effects of aspirin[149] and inhibits platelet recruitment and aggregation in the absence of PGI_2 or NO. For example, in vitro PGI_2 and NO production from endothelial cells can by inhibited by aspirin and hemoglobin, respectively; however, under these conditions, inhibition of platelet responsiveness by endothelial cells persists[149]. Furthermore, CD39-deficient mice have an increased spontaneous thrombotic tendency compared to wild-type mice[151]. Thus, endothelial ecto-ADPase plays an important role in the regulation of hemostasis and platelet-mediated thrombosis independent of PGI_2 or NO.

Consequences of endothelial dysfunction

Exposure to the subendothelium

Under normal conditions, unstimulated platelets do not adhere to intact endothelial cells; however, when normal endothelial function is impaired (endothelial dysfunction) or the endothelium is absent and the subendothelial matrix exposed (as in deep vessel injury), platelets come into contact with substances that promote adhesion and aggregation. In this context, endothelial dysfunction may play an important role in the pathogenesis of certain disease states, including atherosclerosis, hypercholesterolemia, hypertension, diabetes mellitus, and hyperhomocysteinemia. Some of the effects of endothelial dysfunction and subendothelial matrix exposure specifically leading to increased platelet adhesion and aggregation are discussed next.

von Willebrand factor
von Willebrand factor (vWF) is a polymeric glycoprotein that promotes platelet adhesion and thrombus formation at sites of endothelial dysfunction or vascular injury[152,153], especially under conditions of high shear rates. von Willebrand factor is produced by endothelial cells[154], platelets[155], and megakaryocytes[156]. Endothelial cells synthesize vWF, the majority of which is secreted constitutively, either abluminally into the subendothelial matrix or luminally into the circulation. Constitutively secreted vWF is synthesized at a steady-state rate that is not altered by stimulation[157] and contains both pro-vWF and mature subunits. In contrast, endothelial cells stimulated with the calcium ionophore A23187 or thrombin secrete vWF that consists of only large multimers of mature subunits, released from storage in the Weibel–Palade bodies[158,159]. These large multimers of vWF preferentially bind to the extracellular matrix and fibrin. Similarly, platelets and megakaryocytes also contain large multimers of vWF, which are stored in their α-granules[160,161].

Cambridge Books Online © Cambridge University Press, 2009

Released vWF interacts with both the GP Ib–X–V and GP IIb–IIIa receptors on platelets. By binding to subendothelial collagen and glycosaminoglycans, vWF then tethers platelets to the vascular matrix. This interaction initiates platelet adhesion and activation of the GP IIb–IIIa complex[162–165]. The GP IIb-IIIa complex promotes irreversible platelet adhesion as well as platelet spreading at the site of injury, thus allowing additional platelets to be recruited to the growing thrombus.

The importance of vWF in thrombosis is evident in patients with various types of von Willebrand disease (vWD). These patients have decreased platelet adhesion to the subendothelium, which can subsequently be reversed by administration of exogenous vWF[166,167]. In addition, the involvement of vWF in endothelial dysfunction has been established. For example, Fuster and colleages showed that pigs with vWD on a high fat and cholesterol diet are less likely to develop atherosclerosis of the abdominal aorta than normal pigs[168]. In addition, vWF-deficient mice have prolonged bleeding times and increased bleeding events when compared to wild-type mice[169]. Furthermore, in disease states associated with endothelial dysfunction, such as diabetes mellitus and hypertension, plasma vWF levels are markedly elevated[170,171].

Plasminogen activator inhibitor

The endothelium regulates fibrinolysis through the release of several factors, including tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1). Tissue-type plasminogen activator is synthesized and secreted by endothelial cells[172,173] and converts inactive plasminogen to plasmin, which degrades fibrin[174]. Release of t-PA from endothelial cells is augmented by several physiological stimuli, including shear stress[175], and receptor-dependent agonists, such as histamine and thrombin[176–178]. Endothelial cells also express t-PA receptors, which localize plasminogen activation to the endothelial surface. The action of t-PA is principally opposed by PAI-1, which is synthesized by endothelial cells[179] and other cells, such as platelets[180]. Synthesis of PAI-1 is affected by various factors, including inflammatory cytokines[181,182], lipoproteins[183], thrombin[178], and angiotensin II[184]. Under normal conditions, the balance between t-PA and PAI-1 determines the response to thrombus formation in the vessel, which generally favours fibrinolysis. However, when the endothelium is absent (deep vessel injury) or its function is impaired (endothelial dysfunction), the relative preponderance of PAI-1 over t-PA reduces fibrinolysis and promotes thrombosis.

Endothelial dysfunction is associated with several cardiovascular disease states, including atherosclerosis and hypercholesterolemia[33,77], and endothelial control of fibrinolysis is correspondingly altered in these conditions. For example, increased PAI-1 activity was found in young survivors of myocardial infarction and in venous thrombotic states; a reciprocal decrease in t-PA activity was also observed in these conditions in some studies[185]. In addition, increased t-PA antigen levels with decreased t-PA activity and increased PAI-1 activity have been reported in patients with coronary artery disease[186,187] and diabetes mellitus[188]. Increased PAI-1 activity has also been shown to correlate with elevated triglyceride levels[189,190]. Overexpression of PAI-1 in rat carotid arteries increases mural thrombus accumulation[191], while PAI-1-deficient mice have decreased thrombus formation in response to photochemically-induced thrombosis when compared to wild type mice[192]. Furthermore, substances that cause endothelial dysfunction, such as oxidized-LDL and lysophosphatidylcholine, stimulate PAI-1 and inhibit t-PA release from endothelial cells[193].

Thrombin

Antithrombin III present in plasma rapidly inactivates thrombin by forming the thrombin/antithrombin complex, which is subsequently cleared by the liver[194]. The inactivation of thrombin by antithrombin III alone proceeds slowly, but is dramatically increased by heparin, free heparan sulfate, or endothelial cell-bound heparan sulfate[195,196]. Antithrombin III also binds to heparan sulfate in the subendothelium, which catalyses thrombin inactivation[197]. Endothelial cells support the inactivation by antithrombin III of factors IXa, Xa, and XIIa. During aggregation, platelets release platelet factor 4, which binds to glycoaminoglycans and blocks the ability of heparan to accelerate the inactivation of thrombin by antithrombin III[198].

Most normal endothelial surfaces express thrombomodulin, which serves as a binding site for thrombin to activate protein C. Protein C subsequently cleaves and inactivates factors Va and VIIIa[199,200]. Protein S is a cofactor for activated protein C and is required for the expression of protein C's anticoagulant effects[201]. Once thrombin binds to thrombomodulin, the complex is internalized by endocytosis, the attached thrombin is degraded, and the thrombomodulin recycled to the endothelial surface[202]. When the endothelium is minimally damaged, there is evidence to suggest that owing to the high-affinity interaction between thrombin and the endothelial surface, thrombomodulin effectively removes thrombin from plasma and concentrates it on the surface of the endothelial cells downstream of the site of vascular injury. However, with greater extents of injury or endothelial dysfunction, the

Cambridge Books Online © Cambridge University Press, 2009

regulation of thrombin inactivation by antithrombin III and thrombomodulin is decreased, resulting in increased plasma thrombin concentrations. Thrombin is a potent stimulus of platelet aggregation. At low concentrations (less than 1 nM), thrombin stimulates platelets to secrete ADP, serotonin, and fibrinogen, which all enhance platelet aggregation and clot formation. At higher concentrations, thrombin is able to stimulate platelet aggregation by ADP-independent mechanisms[203]. In addition to platelet aggregation, thrombin activates procoagulant factors V and VIII, as well as factor XIII, which catalyses the formation of a covalently crosslinked stable fibrin clot[204,205]. Thrombin can also induce vasoconstriction of the vessel[206]. The mechanism of activation of thrombin and its pathophysiological importance in thrombosis is demonstrated in patients with inherited antithrombin III deficiencies, who have an increased incidence of thrombosis[207,208]. In addition, individuals with autosomal deficiency of protein S, which results in up to an 85% reduction in protein S activity, also have an increased incidence of thrombosis[209], while inherited activated protein C resistance is a major risk factor for venous thrombosis[210]. Furthermore, elevated levels of homocysteine, which are associated with endothelial dysfunction, atherosclerosis, and increased thrombotic events, inhibit thrombomodulin surface expression and protein C activation by endothelial cells[211].

Tissue factor

The main physiological function of tissue factor (TF) is to initiate the coagulation process. Tissue factor binds factor VII in plasma in the presence of calcium and increases the activity of factor X by factor VII. Factor Xa then activates factor VII to VIIa, a process accelerated by endothelial cells and factors VIII and IX[212,213]. Factor Xa generates thrombin that produces local activation of platelets and factors V and VIII[204].

Under normal homeostatic conditions, endothelial cells are devoid of TF. In contrast, the subendothelial matrix possesses TF. Endothelial damage (vessel injury) leads to exposure of plasma and platelets to TF. Expression of TF by vascular smooth muscle cells is rapidly increased by growth factors and thrombin in vitro and by vascular injury in vivo[214]. Furthermore, TF has been identified in atherosclerotic plaques[215], suggesting a role for this proteolipid in the progression of thrombotic complications of cardiovascular disease. In addition to exposure of the subendothelial matrix, endothelial dysfunction can also produce TF expression. Endothelial cells exposed to thrombin or endotoxin markedly increase their TF expression, and the expression of TF initiates fibrin formation[216,217]. Nitric oxide has recently been shown to suppress TF expression by endothelial cells[218], demonstrating yet another antithrombotic action of this vascular effector.

Increased platelet activation

Prostaglandin I_2

Endothelium-derived PGI_2 is a potent vasodilator and inhibitor of platelet aggregation. Physiologically, its actions are counterbalanced by other eicosanoids, such as PGH_2 and TXA_2, with the interaction among these substances determining the response of the vessel. However, when normal endothelial activity is impaired, there is a reduction in the release of bioactive endothelial relaxing factors, such as PGI_2 and NO, and a subsequent increase in the release of endothelial constricting factors (PGH_2 and TXA_2). This shift in prostanoid mediators of vessel tone would be expected to be associated with a shift from an antithrombotic to a prothrombotic state.

Prostaglandin I_2 production is reduced in disease states associated with endothelial dysfunction. For example, in human coronary arteries taken from heart transplant patients, the contribution of PGI_2 towards basal release of relaxing and hyperpolarizing factors was 32% in normal arteries but only 12% in atherosclerotic arteries[219].

Increased production of vasoconstrictor prostaglandins have been shown in diabetes mellitus and hypertension[25,220,221]. Endothelial-dependent vasodilation to acetylcholine in the forearm of patients with essential hypertension is improved by the infusion of the cyclooxygenase inhibitor, indomethacin[222]. In addition, acetylcholine-induced vasodilation of isolated rabbit aorta is improved by treatment with indomethacin[220].

Nitric oxide

Under physiological conditions, endothelium-derived NO plays an important role in the regulation of vascular tone, platelet adhesion, platelet aggregation, myocardial contractility, smooth muscle proliferation, and leukocyte adhesion. However, in disease states associated with endothelial dysfunction, the role of NO is altered. There is evidence that diminished endothelial NO production, and, therefore, decreased bioavailable NO, contributes to atherogenesis by allowing unopposed platelet–endothelial interactions and through the loss of NO-mediated inhibition of vascular smooth muscle proliferation[43]. In contrast, excess NO production has been demonstrated in the pathogenesis of sepsis-induced hypotension and myocardial depression[33]. For the purpose of this review, only impairment in endothelial NO production leading to decreased bioavailable NO will be discussed.

Cambridge Books Online © Cambridge University Press, 2009

The normal endothelium-dependent vasodilator response to intracoronary acetylcholine infusion (via NO release) is converted to a vasoconstrictor response in patients with early or advanced coronary atherosclerosis[223]. However, the vasodilator response to nitroglycerin, a non-endothelium-dependent nitrovasodilator, is unaltered[223]. In addition, endothelium-dependent coronary dilation is lost in hypercholesterolemia before the development of atherosclerosis[224], while impaired flow-mediated dilation has been demonstrated in the brachial arteries of children with familial hypercholesterolemia[225]. Furthermore, the vasodilator responses to stimuli for NO release, such as serotonin[60], substance P[226], and shear stress[227], are impaired in coronary artery disease[228]. Thus, these findings suggest impaired action of NO.

Impaired endothelium-derived NO is also associated with increased platelet adhesion and platelet aggregation. For example, platelets from diabetic subjects demonstrate increased adhesiveness and aggregation[229]. In addition, patients with coronary artery disease exhibit an increased extent of aggregation when compared to age-matched control subjects[230]. Furthermore, patients with acute coronary syndromes also have impaired platelet production of NO[231], which may act in concert with decreased endothelium-derived NO to contribute to the development of coronary atherothrombosis.

Impaired nitric oxide bioavailability

The most important mechanism of impaired endothelium-derived NO bioactivity is increased oxidative stress. Increased production of reactive oxygen species and oxidative modification of lipoproteins account for the majority of oxidative stress in the vessel.

The superoxide anion is produced by a number of enzymatic sources in vascular cells under physiological conditions as a consequence of normal oxidative metabolism[65]. Increased superoxide anion production has been documented in a number of disease states, including hypercholesterolemia[232], diabetes mellitus[233], and hypertension[234]. Enhancing superoxide dismutase activity or inhibiting superoxide anion production is associated with improved endothelium-dependent vasodilator function in atherosclerosis[232]. In addition, patients with coronary atherosclerosis administered ascorbic acid, a potent scavenger of superoxide anion, demonstrate improved flow-mediated brachial dilation[235].

Superoxide anion has also been shown to stimulate platelet aggregation. For example, pyrogallol (a generator of superoxide anion) induces platelet aggregation in vitro, which is completely abolished by superoxide scavengers[236]. In addition, coronary artery disease patients receiving ascorbic acid have shown a reduction in both platelet adhesiveness and platelet aggregation when compared to placebo controls[237].

Superoxide anion interferes with NO, thus limiting its biological activity[238,239]. Nitric oxide and superoxide anion may interact in a diffusion-limited process to form peroxynitrite ($ONOO^-$). Peroxynitrite not only decreases endothelium-derived NO bioavailability, but also initiates lipid peroxidation and oxidization of critical thiols or tyrosine residues[240]. The superoxide anion also undergoes dismutation to hydrogen peroxide, which is subsequently reduced to the hydroxyl radical[241]. Hydrogen peroxide has been shown to mediate endothelial cell injury[242] and impair endothelial production of NO from cultured cells[243].

Lipid peroxidation

The formation of oxidized low-density lipoproteins (ox-LDL) impairs the action of endothelium-derived NO and other endothelial functions by several mechanisms. Oxidized-LDL promotes the recruitment of inflammatory cells to the vessel wall, which may increase local production of reactive oxygen species[244–246]. Oxidized-LDL is also cytotoxic to the endothelial cell and may either directly inactivate NO[247] or decrease eNOS protein levels in endothelial cells[248]. Products of lipid peroxidation, including lysophosphatylcholine, may interfere with signal transduction and receptor-dependent stimulation of NOS activity[249,250], as well as activation of guanylyl cyclase[251].

Increased oxidative stress as a mechanism for endothelial dysfunction is supported by drug therapies directed against LDL oxidation, such as α-tocopherol. For example, α-tocopherol therapy has been shown to preserve endothelium-derived NO function via inhibition of superoxide anion production and cell-mediated LDL oxidation[65].

Other mechanisms for impaired nitric oxide bioavailability

Several other mechanisms exist by which endothelium-derived NO bioavailability can be decreased. These include alterations in NOS expression or NO signalling, reduced availability of the NOS substrate L-arginine, and decreased availability of NOS cofactors, such as tetrahydrobiopterin[252]. However, a detailed discussion of these mechanisms is beyond the scope of this overview[252a].

Ecto-ADPase

Ecto-ADPase is responsible for the enzymatic degradation of ATP and ADP to AMP, which is subsequently converted into the anti-aggregatory substance, adenosine. Ecto-

Cambridge Books Online © Cambridge University Press, 2009

ADPase inhibits platelet recruitment and aggregation in the absence of PGI_2 and NO, and, thus, plays an important role in the regulation of hemostasis and thrombosis. As with PGI_2 and NO, the activity of ecto-ADPase is reduced by endothelial dysfunction.

When endothelial cells are activated by inflammatory cytokines, the activity of ecto-ADPase (CD39) decreases compared with unstimulated cells[253]. This decreased activity has been attributed to inactivation of the enzyme by oxidative stress, and can partially be reversed by antioxidants[151]. In addition, CD39-deficient mice demonstrate substantial fibrin deposition in various tissues. As the activity of ecto-ADPase in these animals is markedly decreased, this pathology suggests impairment in vascular thromboregulation. CD39-deficient mice also demonstrate increased bleeding times, which is contrary to this hypothesis of impairment in thromboregulation. This effect appears to be due to desensitization of the platelet purinergic receptor, since these mice lack both in vivo and in vitro platelet responsiveness to ADP[151]. This lack of platelet responsiveness can be reversed by administration of soluble nucleoside diphosphatase (apyrase).

Pathological conditions associated with endothelial dysfunction

Atherosclerosis

Endothelium-mediated vasodilation is attenuated in atherosclerotic human coronary arteries[254]. Human epicardial arteries dilate in vivo in response to endothelium-dependent agents, such as acetylcholine[223,255], substance P[256], serotonin[60], and histamine[77,257,258], and dilation is impaired in atherosclerotic coronary arteries[77,258]. This impairment in endothelium-dependent vasodilation may be a consequence of decreased production or impaired bioavailability of NO[254]. Furthermore, diminished NO production at atherosclerotic sites may promote atherogenesis by allowing unopposed platelet activation and adhesion of platelets and leukocytes[33,259].

Progressive impairment of endothelial function within the coronary circulation has been observed in patients. For example, impaired endothelium-dependent vasodilation is observed not only in advanced atherosclerotic lesions but also in arteries with minor irregularities[223,260]. This observation also suggests that endothelial dysfunction occurs early in the course of atherosclerosis. The loss of flow-mediated vasodilation in atherosclerotic segments may favor vasoconstriction at sites where vasodilation could ordinarily occur with an increase in flow[223]. Such an impaired response to increased flow may, in part, explain the abnormal vasomotor responses, and, thus, the associated clinical symptoms, accompanying physiological stimuli, such as exercise, mental stress, or exposure to cold environments[261].

Hypercholesterolemia is a well-known risk factor for atherosclerosis and coronary artery disease. Endothelial dysfunction secondary to hypercholesterolemia may be involved in the early pathogenesis of the disease process[224]. Endothelium-dependent vasodilation to serotonin and catecholamines is impaired by hypercholesterolemia in a porcine model[262]. In addition, hypercholesterolemia impairs endothelium-dependent vasodilator responses to aggregating platelets[77]. In humans, hypercholesterolemia impairs vasodilation in resistance vessels[263], which can be acutely improved by infusion of L-arginine.

Hypertension

In animal models of hypertension, endothelium-dependent vasodilation is reduced[264]. However, this endothelial dysfunction varies depending upon the model of hypertension studied. For example, in aortas from spontaneously hypertensive rats the release of endothelium-derived contracting factors in response to serotonin, ADP, and arachidonic acid is increased and explains the inability of the endothelium to attenuate contractions induced by aggregating platelets[259,264]. There is also an increased endothelium-dependent release of prostaglandin H_2 in this model when aortas are exposed to acetylcholine[265]. This response is associated with an enhanced expression of cyclooxygenase-1. In addition, the diminished endothelium-dependent vasodilation to acetylcholine in the forearm of patients with essential hypertension is partially restored by indomethacin[222]. In contrast, in Dahl salt-sensitive hypertensive rats the impairment of endothelium-derived vasodilation is not due to the release of endothelium-derived contracting factors, but decreased responsiveness to NO, as direct vasorelaxant effects of nitrovasodilators are reduced[266]. Furthermore, patients with hypertension have reduced endothelium-derived NO release under basal conditions or during activation of the endothelium[267]; this effect was subsequently shown to be a consequence of impaired NO synthesis or release of NO from the endothelium rather than reduced availability of substrate for NO production[268].

Diabetes mellitus

Endothelium-dependent vasodilation is also impaired in models of diabetes mellitus. Exposure of isolated vascular tissue to high glucose concentrations in vitro produces impairment in the endothelium-dependent vasodilation to acetylcholine[220]. In contrast, the vasodilatory response

Cambridge Books Online © Cambridge University Press, 2009

to A23187 and sodium nitroprusside was unaltered. In addition, the endothelium-dependent vasodilator response is decreased in animals rendered diabetic by alloxan and streptozotocin treatment[269,270]. In diabetic rabbits exposed to high glucose concentrations, impaired endothelium-dependent vasodilation was accompanied by an increased production of vasoconstrictor prostaglandins[220], which could be reversed by inhibitors of prostaglandin synthesis and specific antagonists of prostaglandin H_2/TXA_2.

Impaired endothelium-dependent vasodilation has also been demonstrated in patients with type I diabetes in the absence of clinical complications[271–273]; however, not all studies have shown consistent dysfunction in these patients[274,275]. Similar variability exists in the endothelium-dependent vasodilator responses of patients with type II diabetes[276].

Hyperhomocysteinemia

In epidemiological studies, hyperhomocysteinemia has been associated with premature peripheral artery disease, cerebrovascular disease, and coronary artery disease, independent of the effects of factors such as hyperlipidemia, cigarette smoking, and hypertension[277,278]. Addition of homocysteine to isolated endothelial cells induces functional abnormalities in the release of endothelium-derived NO[279] and detachment of cells[280]. In addition, methionine-induced moderate hyperhomocysteinemia in non-human primates leads to abnormal vasomotor activity[281].

In humans, homozygous homocystinuria is associated with markedly accelerated atherosclerosis and thrombosis, and with endothelial dysfunction in children as young as 4 years old[282]. Furthermore, subjects with no known vascular risk factors other than hyperhomocysteinemia demonstrate impaired arterial endothelium-dependent flow-mediated vasodilation[283]. In this group, hyperhomocysteinemia was not associated with renal failure or deficiencies in folate or vitamin B12. Treatment with folate has been shown to restore endothelial dysfunction during a methionine challenge in healthy volunteers without affecting the rise in plasma homocysteine levels[284]. Hyperhomocysteinemia may also be associated with early arterial injury in adults[285].

Several possible mechanisms exist whereby hyperhomocysteinemia may account of impaired endothelium-dependent vasodilation. These include, increased oxidative stress[286] altered expression of eNOS[287], and suppression of cellular glutathione peroxidase[288].

Hyperhomocysteinemia also effects platelet activation. Homocystinuric patients have abnormally high TXA_2 biosynthesis[289], which is associated with increased platelet activation. In addition, homocysteine rapidly auto-oxidizes in plasma and as a consequence reactive oxygen species, such as superoxide anion, the hydroxyl radial, and hydrogen peroxide are formed[290]. The hydroxyl radical subsequently initiates lipid peroxidation. Di Minno and colleagues demonstrated that short-term administration of probucol, which prevents the oxidative modification of low-density lipoproteins, to homocystinuric patients results in a 40 to 60% decrease in TXA_2 biosynthesis, while having no effects on TXA_2 biosynthesis of normal volunteers[291]. The decrease in TXA_2 biosynthesis was not correlated with either plasma homocysteine levels or plasma cholesterol levels.

Animal models of endothelial dysfunction

Several animal models exist for the investigation of endothelial dysfunction and its relationship to thrombosis and atherosclerosis. These models allow for the study of the mechanisms of platelet activation and interaction with the vessel wall. One of the major models utilized is the Folts coronary thrombosis model of cyclic flow variations (CFVs), which was first described in 1974[292]. This model is used to study platelet aggregation and thrombosis in stenosed arteries with endothelial or medial damage. The CFVs produced are due to the repetitive accumulation and dislodgement of platelet aggregates at the site of endothelial damage associated with arterial stenosis[293–295]. The Folts coronary thrombosis model is created by exposing the heart and dissecting free either the left anterior descending or left circumflex artery from the surrounding tissue. A Doppler flow probe is then placed in the proximal portion of the exposed artery. Endothelial damaged is then produced in the artery, usually by pinching the artery several times with cushioned forceps, just distal to the flow probe. A device (plastic sleeve) is then placed around the artery at the site of endothelial damage and a fixed amount of constriction is produced. Although originally performed acutely in open chested dogs and rabbits the model has been adapted for use in chronic, awake, and unsedated animals[293]. Cyclic flow variations can be abolished by agents that inhibit platelet adhesion and aggregation, such as sodium nitroprusside[296], nitroglycerin[297], TXA_2 or serotonin receptor antagonists[293], aspirin[298], and GP IIb-IIIa receptor antagonists[299,300]. In addition to the Folts coronary thrombosis model, CFVs have been demonstrated in patients undergoing coronary angioplasty[301].

Some other models of endothelial damage and thrombosis are the electrical injury model and the copper coil model. Electrical injury produces endothelial damage by establishing a positive electrical potential on the inner surface of an otherwise normal artery. If sufficient electri-

Cambridge Books Online © Cambridge University Press, 2009

cal potential is supplied, thrombosis occurs[302]. The copper coil model involves the placing of a small metal coil into the left coronary artery of an anesthetized animal via a large-bore catheter inserted in either the carotid or femoral artery[303]. After placement, thrombus formation occurs rapidly and complete arterial occlusion usually occurs within 10 to 20 minutes. Thus, this model has been utilized to study thrombolytic agents[304,305]. However, one of the major problems associated with the above models, at least in the context of endothelial dysfunction, is that they all produce severe endothelial damage. Thus, they represent more accurately models of primary thrombus formation. Other models or variations of the existing ones that are capable of evaluating the role of endothelial dysfunction in thrombosis and atherosclerosis are clearly necessary.

This issue has, in part, been addressed by the development of genetic models with targeted gene disruptions that lead to endothelial dysfunction. Apolipoprotein E (apoE) is a 34 kDa glycoprotein that serves as a ligand for the receptor-mediated clearance of several classes of lipoproteins from plasma, including, very low density lipoproteins, high density lipoproteins, cyclomicrons, and lipoprotein remnants[306]. Thus, targeted disruption of the apoE gene leads to accumulation of plasma cholesterol: homozygous deficiency in apoE (apo$E^{-/-}$) is associated with hypercholesterolemia and spontaneous atherosclerosis[307–309]. Endothelial dysfunction has also been described in apo$E^{-/-}$ mice. Yang and colleagues showed that 7.5-month-old apo$E^{-/-}$ mice develop increased arterial pressure and impaired endothelial function in addition to hypercholesterolemia and atherosclerosis[310]. In this study, endothelial dysfunction was evident by decreased cutaneous blood perfusion on the dorsal side of one hind paw after topical application of mustard oil (NO-mediated vasodilation). In contrast, the same study demonstrated that 6-week-old apo$E^{-/-}$ mice, which were hypercholesterolemic but without atherosclerosis, did not exhibit abnormal arterial pressures or endothelial dysfunction. This result implies that hypertension and endothelial dysfunction are primarily attributed to atherosclerosis, and that NO-mediated vasodilation is diminished with age, at least in this model.

Impaired endothelium-derived NO production / bioavailability is one of the main features of endothelial dysfunction, and has been shown to occur in atherosclerosis, hypertension, and diabetes mellitus. Disruption of the gene encoding eNOS in mice produces hypertension and lack of endothelium-dependent vasodilation by acetylcholine. In addition, mice with transgenic overexpression of eNOS are hypotensive[311]. These findings suggests that eNOS mediates basal release of endothelium-derived NO[312]. However, atherosclerosis has not been reported in eN$OS^{-/-}$ animals.

Hypercholesterolemia is also associated with endothelial dysfunction and is a risk factor for atherosclerosis and thrombosis. Mice heterozygous for a deficiency in the cystathione β-synthase ($CBS^{+/-}$) gene have mild hyperhomocysteinemia[313]. In addition, we have recently shown that $CBS^{+/-}$ mice have attenuated aortic relaxation to the endothelium-dependent agonist, acetylcholine, as well as paradoxical vasoconstriction of the mesenteric microcirculation to methacholine, bradykinin, and A23187[314]. These results are consistent with endothelial dysfunction induced by mild hyperhomocysteinemia. Furthermore, the above study demonstrated that $CBS^{+/-}$ mice have increase superoxide anion production, suggesting that mild hyperhomocysteinemia contributes to endothelial dysfunction by increasing oxidant stress and depleting bioavailable NO.

Glutathione peroxidase-1 (GPx1) catalyses the conversion of hydrogen peroxide and lipid peroxides to water and lipid metabolites, respectively[315]; thus it plays an important role in inhibiting mitochondrial reactive oxygen species production, thereby protecting the animal from oxidative stress. Mice lacking mitochondrial GPx ($GPx^{-/-}$) have increased mitochondrial hydrogen peroxide production, reduced mitochondrial respiratory rates, decreased mitochondrial power output indices and increased levels of lipid peroxides[316]. In addition, these animals display a growth deficiency at 8 months of age. Furthermore, work in our laboratory has demonstrated that $GPx^{+/-}$ and $GPx^{-/-}$ mice have increased oxidative stress when compared to wild type mice[317], and that the mesenteric microvasculature of $GP^{-/-}$ mice paradoxically constricts in response to the vasodilator β-methacholine[318]. This response is reversed by treatment with L-2-oxothiazolidine-4-carboxylate (OTC), which increases the intracellular thiol reducing potential. These results suggest that oxidant stress plays a role in this model of endothelial dysfunction.

Modulation of platelet aggregation by platelet products

Antiplatelet

Nitric oxide

In addition to endothelium-derived NO, which effects vasodilation and inhibits platelet aggregation, NO can also be produced directly by platelets. Once activated, platelets stimulate further thrombus formation and recruitment of

Cambridge Books Online © Cambridge University Press, 2009

additional platelets by releasing ADP and serotonin, producing TXA_2, and promoting surface thrombin generation. However, this activation is somewhat opposed by the release of platelet-derived NO. Platelets contain both cNOS and iNOS[231,319], and upon activation, NO is produced within the context of aggregation. This platelet-derived NO appears to be involved in the regulation of platelet function. For example, systemic infusion of the NOS inhibitor L-NMMA causes a reduction in bleeding time without a change in vessel tone[63], and enhances platelet reactivity to various platelet agonists[320]. We have previously demonstrated that platelet-derived NO only modestly inhibits the primary aggregation response; however, NO released from activated platelets markedly inhibits platelet recruitment. Thus, this mechanism may act to limit the size of an arterial thrombus[116].

In parallel with impaired endothelium-derived NO responsiveness in atherosclerosis, platelet-derived NO production is also decreased in this disease state. We have demonstrated that patients with acute myocardial infarction and unstable angina have decreased platelet-derived NO when compared to normal subjects[231]. In addition, low platelet NO production was independently associated with the presence of acute coronary syndromes. Furthermore, this finding was not attributed to concurrent medical therapy of heparin, nitroglycerin, or aspirin, or to other clinical or coronary risk factors.

Pro-aggregatory

Platelets also secrete a number of substances which enhance platelet responsiveness to primary agonists. Adenosine 5′-diphosphate and serotonin are secreted from the dense granules, while TXA_2 is produced from arachidonic acid. Each of these substances independently produces platelet aggregation; however, when released from the platelet upon stimulation, they act in an autocrine fashion to potentiate the aggregation response.

Conclusion

Under normal conditions the vascular endothelium either synthesizes or converts substances that regulate vascular tone. In addition, the endothelium, through PGI_2, NO, and ecto ADPase, inhibits platelet adhesion to the vessel, activation, and aggregation. By balancing these processes, the endothelium plays an important role in the maintenance of perfusion and hemostasis.

However, the response of the endothelium can be altered, either by its removal in deep vessel injury or by dysfunctional changes associated with disease states, such as atherosclerosis, hypertension, diabetes mellitus, and hyperhomocysteinemia. Endothelial dysfunction leads to a decrease in the synthesis of the vasodilator and antiplatelet agent PGI_2, which subsequently is accompanied by an increased platelet production of the vasoconstrictors and platelet agonists PGH_2/TXA_2. In addition, the potent vasodilatory and antiplatelet effects of NO are reduced owing to decreased NO bioavailability by mechanisms such as, increased oxidative stress and lipid peroxidation. Thus, all of these factors contribute to the pro-thrombotic state associated with endothelial dysfunction and demonstrate the pivotal role of platelets in the pathogenesis of vascular disease.

References

1 Moncada S, Vane JR. Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A2, and prostacyclin. *Pharmacol Rev* 1978; 30: 293–331.

2 Baenziger NL, Force LE, Becherer PR. Histamine stimulates prostacyclin synthesis in cultured human umbilical vein endothelial cells. *Biochem Biophys Res Commun* 1980; 92: 1435–1440.

3 Cramer EB, Pologe L, Pawlowski NA, Cohn ZA, Scott WA. Leukotriene C promotes prostacyclin synthesis by human endothelial cells. *Proc Natl Acad Sci USA* 1983; 80: 4109–4113.

4 D'Humieres S, Russo-Marie F, Vargaftig BB. PAF-acether-induced synthesis of prostacyclin by human endothelial cells. *Eur J Pharmocol* 1986; 131: 13–19.

5 Hong SL. Effect of bradykinin and thrombin on prostacyclin synthesis in endothelial cells from calf and pig aorta and human umbilical cord vein. *Thromb Res* 1980; 18: 787–795.

6 Noll G, Luscher TF. The endothelium in acute coronary syndromes. *Eur Heart J* 1998; 19: C30–C38.

7 Pearson JD, Slakey LL, Gordon JL. Stimulation of prostaglandin production through purinoceptors on cultured porcine endothelial cells. *Biochem J* 1983; 214: 273–276.

8 Weksler BB, Ley CW, Jaffe EA. Stimulation of endothelial cell prostacyclin production by thrombin, trypsin, and the ionophore A23187. *J Clin Invest* 1978; 62: 923–930.

9 Makrides SC, Ryan US. Overview of the endothelium. In Loscalzo J, Schafer AI, eds. *Thrombosis and hemorrhage*, 2nd edn. Baltimore: Williams and Wilkins; 1998: 295–306.

10 Marcus AJ, Weksler BB, Jaffe EA, Broekman MJ. Synthesis of prostacyclin from platelet-derived endoperoxides by cultured human endothelial cells. *J Clin Invest* 1980; 66: 979–986.

11 Bhagyalakshmi A, Frangos JA. Mechanism of shear-induced prostacyclin production in endothelial cells. *Biochem Biophys Res Commun* 1989; 158: 31–37.

12 Crutchley DJ, Ryan JW, Ryan US, Fisher GH, Paul SM. Effects

Cambridge Books Online © Cambridge University Press, 2009

of bradykinin and its homologs on the metabolism of arachidonate by endothelial cells. *Adv Exp Med Biol* 1983; 156: 527–532.
13 Endo H, Akahoshi T, Kashiwazaki S. Additive effects of IL-1 and TNF on induction of prostacyclin synthesis in human vascular endothelial cells. *Biochem Biophys Res Commun* 1988; 156: 1007–1014.
14 Grabowski EF, Jaffe EA, Weksler BB. Prostacyclin production by cultured endothelial cell monolayers exposed to step increases in shear stress. *J Lab Clin Med* 1985; 105: 36–43.
15 Nollert MU, Hall ER, Eskin SG, McIntire LV. The effect of shear stress on the uptake and metabolism of arachidonic acid by human endothelial cells. *Biochim Biophys Acta* 1989; 1005: 72–78.
16 Rossi V, Breviario F, Ghezzi P, Dejana E, Mantovani A. Prostacyclin synthesis induced in vascular cells by interleukin-1. *Science* 1985; 229: 174–176.
17 Kukovetz WR, Holzmann S, Wurm A, Poch G. Prostacyclin increases cAMP in coronary arteries. *J Cyclic Nucleotide Res* 1979; 5: 469–476.
18 Marcus A. Multicellular eicosanoid and other metabolic interactions of platelets and other cells. In Colman RW, Hirsh J, Marder VJ, Salzman EW, eds. *Hemostasis and thrombosis: basic principles and clinical practice*, 3rd Edn. Philadelphia: J.B. Lippincott Company: 1994: 590–602.
19 Gorman RR, Bunting S, Miller OV. Modulation of human platelet adenylate cyclase by prostacyclin (PGX). *Prostaglandins* 1977; 13: 377–388.
20 Kinlough-Rathbone RL, Packham MA, Mustard JF. The effect of prostaglandin E1 on platelet function in vitro and in vivo. *Br J Haematol* 1970: 19: 559–571.
21 Tateson JE, Moncada S, Vane JR. Effects of prostacyclin (PGX) on cyclic AMP concentrations in human platelets. *Prostaglandins* 1977; 13: 389–397.
22 Marcus AJ, Safier LB. Thromboregulation: multicellular modulation of platelet reactivity in hemostasis and thrombosis. *Faseb J* 1993; 7: 516–522.
23 Body SC. Platelet activation and interactions with the microvasculature. *J Cardiovasc Pharmacol* 1996; 27: S13–S25.
24 Dusting GJ, MacDonald PS. Prostacyclin and vascular function: implications for hypertension and atherosclerosis. *Pharmacol Ther* 1990; 48: 323–344.
25 Luscher TF, Boulanger CM, Dohi Y, Yang ZH. Endothelium-derived contracting factors. *Hypertension* 1992; 19: 117–130.
26 Cohen RA. Endothelium-derived vasoactive factors. In: Loscalzo J, Schafer AI, eds. *Thrombosis and hemorrhage*, Second Edition. Baltimore: Williams and Wilkins; 1998, 387–404.
27 Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 1980; 288: 373–376.
28 Feelisch M, te Poel M, Zamora R, Deussen A, Moncada S. Understanding the controversy over the identity of EDRF. *Nature* 1994; 368: 62–65.
29 Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci USA* 1987; 84: 9265–9269.
30 Ignarro LJ, Buga GM, Byrns RE, Wood KS, Chaudhuri G. Endothelium-derived relaxing factor and nitric oxide possess identical pharmacologic properties as relaxants of bovine arterial and venous smooth muscle. *J Pharmacol Exp Ther* 1988; 246: 218–226.
31 Myers PR, Guerra R, Jr., Harrison DG. Release of NO and EDRF from cultured bovine aortic endothelial cells. *Am J Physiol* 1989; 256: H1030–H1037.
32 Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987; 327: 524–526.
33 Loscalzo J, Welch G. Nitric oxide and its role in the cardiovascular system. *Prog Cardiovasc Dis* 1995; 38: 87–104.
34 Palmer RM, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 1988; 333: 664–666.
35 Leone AM, Palmer RM, Knowles RG, Francis PL, Ashton DS, Moncada S. Constitutive and inducible nitric oxide synthases incorporate molecular oxygen into both nitric oxide and citrulline. *J Biol Chem* 1991; 266: 23790–23795.
36 Nathan C. Nitric oxide as a secretory product of mammalian cells. *Faseb J* 1992; 6: 3051–3064.
37 Yui Y, Hattori R, Kosuga K et al. Calmodulin-independent nitric oxide synthase from rat polymorphonuclear neutrophils. *J Biol Chem* 1991; 266: 3369–3371.
38 Bredt DS, Ferris CD, Snyder SH. Nitric oxide synthase regulatory sites. Phosphorylation by cyclic AMP-dependent protein kinase, protein kinase C, and calcium/calmodulin protein kinase; identification of flavin and calmodulin binding sites. *J Biol Chem* 1992; 267: 10976–10981.
39 McMillan K, Bredt DS, Hirsch DJ, Snyder SH, Clark JE, Masters BS. Cloned, expressed rat cerebellar nitric oxide synthase contains stoichiometric amounts of heme, which binds carbon monoxide. *Proc Natl Acad Sci USA* 1992; 89: 11141–11145.
40 Nishida K, Harrison DG, Navas JP et al. Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. *J Clin Invest* 1992; 90: 2092–2096.
41 Stuehr DJ, Kwon NS, Nathan CF. FAD and GSH participate in macrophage synthesis of nitric oxide. *Biochem Biophys Res Commun* 1990; 168: 558–565.
42 Tayeh MA, Marletta MA. Macrophage oxidation of L-arginine to nitric oxide, nitrite, and nitrate. Tetrahydrobiopterin is required as a cofactor. *J Biol Chem* 1989; 264: 19654–19658.
43 Dinerman JL, Lowenstein CJ, Snyder SH. Molecular mechanisms of nitric oxide regulation. Potential relevance to cardiovascular disease. *Circ Res* 1993; 73: 217–222.
44 Bredt DS, Snyder SH. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc Natl Acad Sci USA* 1990; 87: 682–685.

Cambridge Books Online © Cambridge University Press, 2009

45 Mayer B, Schmidt K, Humbert P, Bohme E. Biosynthesis of endothelium-derived relaxing factor: a cytosolic enzyme in porcine aortic endothelial cells Ca^{2+}-dependently converts L-arginine into an activator of soluble guanylyl cyclase. *Biochem Biophys Res Commun* 1989; 164: 678–685.
46 Ignarro LJ. Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein. *Circ Res* 1989; 65: 1–21.
47 Stamler JS, Singel DJ, Loscalzo J. Biochemistry of nitric oxide and its redox-activated forms [see comments]. *Science* 1992; 258: 1898–1902.
48 Ignarro LJ. Signal transduction mechanisms involving nitric oxide. *Biochem Pharmacol* 1991; 41: 485–490.
49 Wolin MS, Wood KS, Ignarro LJ. Guanylate cyclase from bovine lung. A kinetic analysis of the regulation of the purified soluble enzyme by protoporphyrin IX, heme, and nitrosyl-heme. *J Biol Chem* 1982; 257: 13312–13320.
50 Ignarro LJ, Adams JB, Horiwitz PM, Wood KS. Activation of soluble guanylate cyclase by NO-hemoproteins involves NO-heme exchange. Comparison of heme-containing and heme-deficient enzyme forms. *J Biol Chem* 1986; 261: 4997–5002.
51 Bolotina VM, Najibi S, Palacino JJ, Pagano PJ, Cohen RA. Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* 1994; 368: 850–853.
52 Maurice DH, Haslam RJ. Molecular basis of the synergistic inhibition of platelet function of nitrovasodilators and activators of adenylate cyclase: inhibition of cyclic AMP breakdown by cyclic GMP. *Mol Pharmacol* 1990; 37: 671–681.
53 Mellion BT, Ingnarro LJ, Ohlstein EH, Pontecorvo EG, Hyman AL, Kadowitz PJ. Evidence for the inhibitory role of guanosine 3′, 5′-monophosphate in ADP-induced human platelet aggregation in the presence of nitric oxide and related vasodilators. *Blood* 1981; 57: 946–955.
54 Griffith TM, Edwards DH, Davies RL, Harrison TJ, Evans KT. EDRF coordinates the behaviour of vascular resistance vessels. *Nature* 1987; 329: 442–445.
55 Lowenstein CJ, Dinerman JL, Snyder SH. Nitric oxide: a physiologic messenger. *Ann Intern Med* 1994; 120: 227–237.
56 Rees DD, Palmer RM, Schulz R, Hodson HF, Moncada S. Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *Br J Pharmacol* 1990; 101: 746–752.
57 Vallance P, Collier J, Moncada S. Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man [see comments]. *Lancet* 1989; 2: 997–1000.
58 Rees DD, Palmer RM, Moncada S. Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc Natl Acad Sci USA* 1989; 86: 3375–3378.
59 Vita JA, Treasure CB, Yeung AC et al. Patients with evidence of coronary endothelial dysfunction as assessed by acetylcholine infusion demonstrate marked increase in sensitivity to constrictor effects of catecholamines. *Circulation* 1992; 85: 1390–1397.
60 Golino P, Piscione F, Willerson JT et al. Divergent effects of serotonin on coronary-artery dimensions and blood flow in patients with coronary atherosclerosis and control patients [see comments]. *N Engl J Med* 1991; 324: 641–648.
61 Gilligan DM, Guetta V, Panza JA, Garcia CE, Quyyumi AA, Cannon RO, 3rd. Selective loss of microvascular endothelial function in human hypercholesterolemia [see comments]. *Circulation* 1994; 90: 35–41.
62 Meredith IT, Currie KE, Anderson TJ, Roddy MA, Ganz P, Creager MA. Postischemic vasodilation in human forearm is dependent on endothelium-derived nitric oxide. *Am J Physiol* 1996; 270: H1435–H1440.
63 Simon DI, Stamler JS, Loh E, Loscalzo J, Francis SA, Creager MA. Effect of nitric oxide synthase inhibition on bleeding time in humans. *J Cardiovasc Pharmacol* 1995; 26: 339–342.
64 Adams MR, Jessup W, Hailstones D, Celermajer DS. L-arginine reduces human monocyte adhesion to vascular endothelium and endothelial expression of cell adhesion molecules [see comments]. *Circulation* 1997; 95: 662–668.
65 Tomasian D, Keaney JF, Vita JA. Antioxidants and the bioactivity of endothelium-derived nitric oxide. *Cardiovasc Res* 2000; 47: 426–435.
66 Furchgott RF. Role of endothelium in responses of vascular smooth muscle. *Circ Res* 1983; 53: 557–573.
67 Young MA, Vatner SF. Blood flow- and endothelium-mediated vasomotion of iliac arteries in conscious dogs. *Circ Res* 1987; 61: II88–II93.
68 Cowan CL, Cohen RA. Two mechanisms mediate relaxation by bradykinin of pig coronary artery: NO-dependent and -independent responses. *Am J Physiol* 1991; 261: H830–H835.
69 Feletou M, Vanhoutte PM. Endothelium-derived hyperpolarizing factor. *Clin Exp Pharmacol Physiol* 1996; 23: 1082–1090.
70 Mombouli JV, Vanhoutte PM. Endothelial dysfunction: from physiology to therapy. *J Mol Cell Cardiol* 1999; 31: 61–74.
71 Nagao T, Vanhoutte PM. Hyperpolarization as a mechanism for endothelium-dependent relaxations in the porcine coronary artery. *J Physiol (Lond)* 1992; 445: 355–367.
72 Suzuki H, Chen G, Yamamoto Y, Miwa K. Nitroarginine-sensitive and -insensitive components of the endothelium-dependent relaxation in the guinea-pig carotid artery. *Jpn J Physiol* 1992; 42: 335–347.
73 Beny JL, Brunet PC. Neither nitric oxide nor nitroglycerin accounts for all the characteristics of endothelially mediated vasodilation of pig coronary arteries. *Blood Vessels* 1988; 25: 308–311.
74 Feletou M, Vanhoutte PM. Endothelium-dependent hyperpolarization of canine coronary smooth muscle. *Br J Pharmacol* 1988; 93: 515–524.
75 Campbell WB, Gebremedhin D, Pratt PF, Harder DR. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ Res* 1996; 78: 415–423.
76 Komori K, Shimokawa H, Vanhoutte PM. Hypercholesterolemia impairs endothelium-dependent relaxation

Cambridge Books Online © Cambridge University Press, 2009

to aggregating platelets in porcine iliac arteries. *J Vasc Surg* 1989; 10: 318–325.

77 Shimokawa H. Primary endothelial dysfunction: atherosclerosis. *J Mol Cell Cardiol* 1999; 31: 23–37.

78 Chen G, Suzuki H. Some electrical properties of the endothelium-dependent hyperpolarization recorded from rat arterial smooth muscle cells. *J Physiol (Lond)* 1989; 410: 91–106.

79 Chen G, Yamamoto Y, Miwa K, Suzuki H. Hyperpolarization of arterial smooth muscle induced by endothelial humoral substances. *Am J Physiol* 1991; 260: H1888–1892.

80 Van de Voorde J, Vanheel B, Leusen I. Endothelium-dependent relaxation and hyperpolarization in aorta from control and renal hypertensive rats. *Circ Res* 1992; 70: 1–8.

81 Cohen RA, Vanhoutte PM. Endothelium-dependent hyperpolarization. Beyond nitric oxid and cyclic GMP. *Circulation* 1995; 92: 3337–3349.

82 Feletou M, Vanhoutte PM. The alternative: EDHF. *J Mol Cell Cardiol* 1999; 31: 15–22.

83 Nakashima M, Mombouli JV, Taylor AA, Vanhoutte PM. Endothelium-dependent hyperpolarization caused by bradykinin in human coronary arteries. *J Clin Invest* 1993; 92: 2867–2871.

84 Urakami-Harasawa L, Shimokawa H, Nakashima M, Egashira K, Takeshita A. Importance of endothelium-derived hyperpolarizing factor in human arteries [published erratum appears in *J Clin Invest* 1998 Mar 15; 101: 1491]. *J Clin Invest* 1997; 100: 2793–2799.

85 Shimokawa H, Yasutake H, Fujii K et al. The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J Cardiovasc Pharmacol* 1996; 28: 703–711.

86 Olmos L, Mombouli JV, Illiano S, Vanhoutte PM. cGMP mediates the desensitization to bradykinin in isolated canine coronary arteries. *Am J Physiol* 1995; 268: H865–H870.

97 Bauersachs J, Popp R, Hecker M, Sauer E, Fleming I, Busse R. Nitric oxide attenuates the release of endothelium-derived hyperpolarizing factor. *Circulation* 1996; 94: 3341–3347.

88 Takase H, Moreau P, Kung CF, Nava E, Luscher TF. Antihypertensive therapy prevents endothelial dysfunction in chronic nitric oxide deficiency. Effect of verapamil and trandolapril. *Hypertension* 1996; 27: 25–31.

89 Hickey KA, Rubanyi G, Paul RJ, Highsmith RF. Characterization of a coronary vasoconstrictor produced by cultured endothelial cells. *Am J Physiol* 1985; 248: C550–C556.

90 Schmitz-Spanke S, Schipke JD. Potential role of endothelin-1 and endothelin antagonists in cardiovascular diseases [In Process Citation]. *Basic Res Cardiol* 2000; 95: 290–298.

91 Russell FD, Skepper JN, Davenport AP. Endothelin peptide and converting enzymes in human endothelium. *J Cardiovasc Pharmacol* 1998; 31: S19–21.

92 Boulanger C, Luscher TF. Release of endothelin from the porcine aorta. Inhibition by endothelium-derived nitric oxide. *J Clin Invest* 1990; 85: 587–590.

93 Ohlstein EH, Arleth A, Ezekiel M, Horohonich S, Ator MA, Caltabiano MM et al. Biosynthesis and modulation of endothelin from bovine pulmonary arterial endothelial cells. *Life Sci* 1990; 46: 181–188.

94 Ohlstein EH, Storer BL, Butcher JA, Debouck C, Feuerstein G. Platelets stimulate expression of endothelin mRNA and endothelin biosynthesis in cultured endothelial cells. *Circ Res* 1991; 69: 832–841.

95 Yanagisawa M, Kurihara H, Kimura S et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells [see comments]. *Nature* 1988; 332: 411–415.

96 Yanagisawa M, Masaki T. Molecular biology and biochemistry of the endothelins. *Trends Pharmacol Sci* 1989; 10: 374–378.

97 Yoshizumi M, Kurihara H, Morita T et al. Interleukin 1 increases the production of endothelin-1 by cultured endothelial cells. *Biochem Biophys Res Commun* 1990; 166: 324–329.

98 Hori S, Komatsu Y, Shigemoto R, Mizuno N, Nakanishi S. Distinct tissue distribution and cellular localization of two messenger ribonucleic acids encoding different subtypes of rat endothelin receptors. *Endocrinology* 1992; 130: 1885–1895.

99 Ogawa Y, Nakao K, Arai H et al. Molecular cloning of a non-isopeptide-selective human endothelin receptor. *Biochem Biophys Res Commun* 1991; 178: 248–255.

100 Yanagisawa M. The endothelin system. A new target for therapeutic intervention [editorial; comment]. *Circulation* 1994; 89: 1320–1322.

101 Lüscher TF, Wenzel RR, Noll G. Local regulation of the coronary circulation in health and disease: role of nitric oxide and endothelin. *Eur Heart J* 1995; 16: 51–58.

102 Marsault R, Vigne P, Breittmayer JP, Frelin C. Kinetics of vasoconstrictor action of endothelins. *Am J Physiol* 1991; 261: C987–C993.

103 Bassenge E. Control of coronary blood flow by autacoids. *Basic Res Cardiol* 1995; 90: 125–141.

104 Halushka PV, Mais DE, Saussy DL, Jr. Platelet and vascular smooth muscle thromboxane A2/prostaglandin H2 receptors. *Fed Proc* 1987; 46: 149–153.

105 Kato T, Iwama Y, Okumura K, Hashimoto H, Ito T, Satake T. Prostaglandin H2 may be the endothelium-derived contracting factor released by acetylcholine in the aorta of the rat. *Hypertension* 1990; 15: 475–481.

106 Smith WL. The eicosanoids and their biochemical mechanisms of action. *Biochem J* 1989; 259: 315–324.

107 Kroll MH, Schafer AI. Biochemical mechanisms of platelet activation. *Blood* 1989; 74: 1181–1195.

108 Stamler JS, Vaughan DE, Loscalzo J. Synergistic disaggregation of platelets by tissue-type plasminogen activator, prostaglandin E1, and nitroglycerin. *Circ Res* 1989; 65: 796–804.

109 Cooke JP, Stamler J, Andon N, Davies PF, McKinley G, Loscalzo J. Flow stimulates endothelial cells to release a

Cambridge Books Online © Cambridge University Press, 2009

nitrovasodilator that is potentiated by reduced thiol. *Am J Physiol* 1990; 259: H804–H812.
110 Willoughby SR, Chirkova LP, Chirkov YY, Horowitz JD. Multiple agonist induction of aggregation: An approach to examine anit-aggregating effects in vitro. *Platelets* 1996; 7: 329–333.
111 Nishikawa M, de Lanerolle P, Lincoln TM, Adelstein RS. Phosphorylation of mammalian myosin light chain kinases by the catalytic subunit of cyclic AMP-dependent protein kinase and by cyclic GMP-dependent protein kinase. *J Biol Chem* 1984; 259: 8429–8436.
112 Hathaway DR, Konicki MV, Coolican SA. Phosphorylation of myosin light chain kinase from vascular smooth muscle by cAMP- and cGMP-dependent protein kinases. *J Mol Cell Cardiol* 1985; 17: 841–850.
113 Ignarro LJ, Kadowitz PJ. The pharmacological and physiological role of cyclic GMP in vascular smooth muscle relaxation. *Annu Rev Pharmacol Toxicol* 1985; 25: 171–191.
114 Michelson AD, Benoit SE, Furman MI et al. Effects of nitric oxide/EDRF on platelet surface glycoproteins. *Am J Physiol* 1996; 270: H1640–H1648.
115 Lüscher TF, Diederich D, Siebenmann R et al. Difference between endothelium-dependent relaxation in arterial and in venous coronary bypass grafts. *N Engl J Med* 1988; 319: 462–467.
116 Freedman JE, Loscalzo J, Barnard MR, Alpert C, Keaney JF, Michelson AD. Nitric oxide released from activated platelets inhibits recruitment. *J Clin Invest* 1997; 100: 350–356.
117 Radomski MW, Palmer RM, Moncada S. An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc Natl Acad Sci USA* 1990; 87: 5193–5197.
118 Malinski T, Radomski MW, Taha Z, Moncada S. Direct electrochemical measurement of nitric oxide released from human platelets. *Biochem Biophys Res Commun* 1993; 194: 960–965.
119 Chen LY, Mehta JL. Variable effects of L-arginine analogs on L-arginine-nitric oxide pathway in human neutrophils and platelets may relate to different nitric oxide synthase isoforms. *J Pharmacol Exp Ther* 1996; 276: 253–257.
120 Radomski MW, Palmer RM, Moncada S. The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. *Br J Pharmacol* 1987; 92: 639–646.
121 Radomski MW, Palmer RM, Moncada S. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet* 1987; 2: 1057–1058.
122 Pigazzi A, Heydrick S, Folli F, Benoit S, Michelson A, Loscalzo J. Nitric oxide inhibits thrombin receptor-activating peptide-induced phosphoinositide 3-kinase activity in human platelets. *J Biol Chem* 1999; 274: 14368–14375.
123 Quon MJ, Chen H, Ing BL et al. Roles of 1-phosphatidylinositol 3-kinase and ras in regulating translocation of GLUT4 in transfected rat adipose cells. *Mol Cell Biol* 1995; 15: 5403–5411.
124 Toker A, Cantley LC. Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature* 1997; 387: 673–676.
125 Carpenter CL, Auger KR, Duckworth BC, Hou WM, Schaffhausen B, Cantley LC. A tightly associated serine/threonine protein kinase regulates phosphoinositide 3-kinase activity. *Mol Cell Biol* 1993; 13: 1657–1665.
126 Lam K, Carpenter CL, Ruderman NB, Friel JC, Kelly KL. The phosphatidylinositol 3-kinase serine kinase phosphorylates IRS-1. Stimulation by insulin and inhibition by Wortmannin. *J Biol Chem* 1994; 269: 20648–20652.
127 Zhang J, Shattil SJ, Cunningham MC, Rittenhouse SE. Phosphoinositide 3-kinase gamma and p85/phosphoinositide 3-kinase in platelets. Relative activation by thrombin receptor or beta-phorbol myristate acetate and roles in promoting the ligand-binding function of alphaIIbbeta3 integrin. *J Biol Chem* 1996; 271: 6265–6272.
128 Ptasznik A, Traynor-Kaplan A, Bokoch GM. G protein-coupled chemoattractant receptors regulate Lyn tyrosine kinase. Shc adapter protein signalling complexes [published erratum appears in *J Biol Chem* 1995 Oct 13; 270: 24622]. *J Biol Chem* 1995; 270: 19969–19973.
129 Kucera GL, Rittenhouse SE. Human platelets form 3-phosphorylated phosphoinositides in response to alpha-thrombin, U46619, or GTP gamma S. *J Biol Chem* 1990; 265: 5345–5348.
130 Kovacsovics TJ, Bachelot C, Toker A et al. Phosphoinositide 3-kinase inhibition spares actin assembly in activating platelets but reverses platelet aggregation. *J Biol Chem* 1995; 270: 11358–11366.
131 Papapetropoulos A, Garcia-Cardena G, Madri JA, Sessa WC. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J Clin Invest* 1997; 100: 3131–3139.
132 Zeng G, Quon MJ. Insulin-stimulated production of nitric oxide is inhibited by wortmannin. Direct measurement in vascular endothelial cells. *J Clin Invest* 1996; 98: 894–898.
133 Shaul PW, Smart EJ, Robinson LJ et al. Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae. *J Biol Chem* 1996; 271: 6518–6522.
134 Kim I, Kim HG, So JN, Kim JH, Kwak HJ, Koh GY. Angiopoietin-1 regulates endothelial cell survival through the phosphatidylinositol 3′-Kinase/Akt signal transduction pathway [see comments]. *Circ Res* 2000; 86: 24–29.
135 Dimmeler S, Zeiher AM. Akt takes center stage in angiogenesis signaling [editorial; comment]. *Circ Res* 2000; 86: 4–5.
136 Gerber HP, McMurtrey A, Kowalski J et al. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem* 1998; 273: 30336–30343.
137 Fujio Y, Walsh K. Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. *J Biol Chem* 1999; 274: 16349–16354.
138 Dimmeler S. Assmus B, Hermann C, Haendeler J, Zeiher AM. Fluid shear stress stimulates phosphorylation of Akt in human endothelial cells: involvement in suppression of apoptosis. *Circ Res* 1998; 83: 334–341.

Cambridge Books Online © Cambridge University Press, 2009

139 Hermann C, Assmus B, Urbich C, Zeiher AM, Dimmeler S. Insulin-mediated stimulation of protein kinase Akt: A potent survival signaling cascade for endothelial cells. *Arterioscler Thromb Vasc Biol* 2000; 20: 402–409.
140 Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phsophorylation. *Nature* 1999; 399: 601–605.
141 Fulton D, Gratton JP, McCabe TJ et al. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt [published erratum appears in *Nature* 1999 Aug 19; 400: 792]. *Nature* 1999; 399: 597–601.
142 Dimmeler S, Lottspeich F, Brune B. Nitric oxide causes ADP-ribosylation and inhibition of glyceraldehyde-3-phosphate dehydrogenase. *J Biol Chem* 1992; 267: 16771–16774.
143 Gupta S, McArthur C, Grady C, Ruderman NB. Stimulation of vascular Na(+)- K(+)-ATPase activity by nitric oxide: a cGMP-independent effect. *Am J Physiol* 1994; 266: H2146–H2151.
144 Mendelsohn ME, O'Neill S, George D, Loscalzo J. Inhibition of fibrinogen binding to human platelets by S-nitroso-N-acetylcysteine. *J Biol Chem* 1990; 265: 19028–19034.
145 Trepakova ES, Cohen RA, Bolotina VM. Nitric oxide inhibits capacitative cation influx in human platelets by promoting sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase-dependent refilling of Ca^{2+} stores. *Circ Res* 1999; 84: 201–209.
146 Fredholm BB, Abbracchio MP, Burnstock G et al. Nomenclature and classification of purinoceptors. *Pharmacol Rev* 1994; 46: 143–156.
147 Zimmermann H. Nucleotides and cd39: principal modulatory players in hemostasis and thrombosis [news; comment]. *Nat Med* 1999; 5: 987–988.
148 Plesner L. Ecto-ATPases: identities and functions. *Int Rev Cytol* 1995; 158: 141–214.
149 Marcus AJ, Safier LB, Hajjar KA et al. Inhibition of platelet function by an aspirin-insensitive endothelial cell ADPase. Thromboregulation by endothelial cells. *J Clin Invest* 1991; 88: 1690–1696.
150 Kaczmarek E, Koziak K, Sevigny J et al. Identification and characterization of CD39/vascular ATP diphosphohydrolase. *J Biol Chem* 1996; 271: 33116–33122.
151 Enjyoji K, Sevigny J, Lin Y et al. Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation [see comments]. *Nat Med* 1999; 5: 1010–1017.
152 Turitto VT, Weiss HJ, Zimmerman TS, Sussman, II. Factor VIII/von Willebrand factor in subendothelium mediates platelet adhesion. *Blood* 1985; 65: 823–831.
153 Meyer D, Baumgartner HR. Role of von Willebrand factor in platelet adhesion to the subendothelium. *Br J Haematol* 1983; 54: 1–9.
154 Jaffe EA, Hoyer LW, Nachman RL. Synthesis of antihemophilic factor antigen by cultured human endothelial cells. *J Clin Invest* 1973; 52: 2757–2764.
155 Jaffe EA, Hoyer LW, Nachman RL. Synthesis of von Willebrand factor by cultured human endothelial cells. *Proc Natl Acad Sci USA* 1974; 71: 1906–1909.
156 Jeanneau C, Avner P, Sultan Y. Use of monoclonal antibody and colloidal gold in E.M. localization of von Willebrand factor in megakaryocytes and platelets. *Cell Biol Int Rep* 1984; 8: 841–848.
157 Mayadas T, Wagner DD, Simpson PJ. von Willebrand factor biosynthesis and partitioning between constitutive and regulated pathways of secretion after thrombin stimulation. *Blood* 1989; 73: 706–711.
158 Warhol MJ, Sweet JM. The ultrastructural localization of von Willebrand factor in endothelial cells. *Am J Pathol* 1984; 117: 310–315.
159 Wagner DD, Marder VJ. Biosynthesis of von Willebrand protein by human endothelial cells: processing steps and their intracellular localization. *J Cell Biol* 1984; 99: 2123–2130.
160 Sporn LA, Marder VJ, Wagner DD. Inducible secretion of large, biologically potent von Willebrand factor multimers. *Cell* 1986; 46: 185–190.
161 Loesberg C, Gonsalves MD, Zandbergen J et al. The effect of calcium on the secretion of factor VIII-related antigen by cultured human endothelial cells. *Biochim Biophys Acta* 1983; 763: 160–168.
162 Weiss HJ, Hawiger J, Ruggeri ZM, Turitto VT, Thiagarajan P, Hoffmann T. Fibrinogen-independent platelet adhesion and thrombus formation on subendothelium mediated by glycoprotein IIb–IIIa complex at high shear rate. *J Clin Invest* 1989; 83: 288–297.
163 Ideda Y, Handa M, Kawano K et al. The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. *J Clin Invest* 1991; 87; 1234–1240.
164 Ruggeri ZM, De Marco L, Gatti L, Bader R, Montgomery RR. Platelets have more than one binding site for von Willebrand factor. *J Clin Invest* 1983; 72: 1–12.
165 Savage B, Shattil SJ, Ruggeri ZM. Modulation of platelet function through adhesion receptors. A dual role for glycoprotein IIb–IIIa (integrin alpha IIb beta 3) mediated by fibrinogen and glycoprotein Ib-von Willebrand factor. *J Biol Chem* 1992; 267: 11300–11306.
166 Tschopp TB, Weiss HJ, Baumgartner HR. Decreased adhesion of platelets to subendothelium in von Willebrand's disease. *J Lab Clin Med* 1974; 83: 296–300.
167 Weiss HJ, Baumgartner HR, Tschopp TB, Turitto VT, Cohen D. Correction by factor VIII of the impaired platelet adhesion to subendothelium in von Willebrand disease. *Blood* 1978; 51: 267–279.
168 Fuster V, Lie JT, Badimon L, Rosemark JA, Badimon JJ, Bowie EJ. Spontaneous and diet-induced coronary atherosclerosis in normal swine and swine with von Willebrand disease. *Arteriosclerosis* 1985; 5: 67–73.
169 Denis C, Methia N, Frenette PS et al. A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. *Proc Natl Acad Sci USA* 1998; 95: 9524–9529.
170 Kario K, Hoshide S, Matsuo T, Shimada K. [Determinants of

Cambridge Books Online © Cambridge University Press, 2009

endothelial cell damage in the elderly hypertension: assessment by plasma von Willebrand factor]. *Nippon Ronen Igakkai Zasshi* 2000; 37: 393–397.
171 Kessler L, Wiesel ML, Attali P, Mossard JM, Cazenave JP, Pinget M. Von Willebrand factor in diabetic angiopathy. *Diabetes Metab* 1998; 24: 327–336.
172 Loskutoff DJ, Edgington TE. Synthesis of a fibrinolytic activator and inhibitor by endothelial cells. *Proc Natl Acad Sci USA* 1977; 74: 3903–3907.
173 Bykowska K, Levin EG, Rijken DC, Loskutoff DJ, Collen D. Characterization of a plasminogen activator secreted by cultured bovine aortic endothelial cells. *Biochim Biophys Acta* 1982; 703: 113–115.
174 Hoylaerts M, Rijken DC, Lijnen HR, Collen D. Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. *J Biol Chem* 1982; 257: 2912–2919.
175 Diamond SL, Eskin SG, McIntire LV. Fluid flow stimulates tissue plasminogen activator secretion by cultured human endothelial cells. *Science* 1989; 243: 1483–1485.
176 Levin EG, Marzec U, Anderson J, Harker LA. Thrombin stimulates tissue plasminogen activator release from cultured human endothelial cells. *J Clin Invest* 1984; 74: 1988–1995.
177 Hanss M, Collen D. Secretion of tissue-type plasminogen activator and plasminogen activator inhibitor by cultured human endothelial cells: modulation by thrombin, endotoxin, and histamine. *J Lab Clin Med* 1987; 109: 97–104.
178 Gelehrter TD, Sznycer-Laszuk R. Thrombin induction of plasminogen activator-inhibitor in cultured human endothelial cells. *J Clin Invest* 1986; 77: 165–169.
179 Hekman CM, Loskutoff DJ. Endothelial cells produce a latent inhibitor of plasminogen activators that can be activated by denaturants. *J Biol Chem* 1985; 260: 11581–11587.
180 Konkle BA, Schick PK, He X, Liu RJ, Mazur EM. Plasminogen activator inhibitor-1 mRNA is expressed in platelets and megakaryocytes and the megakaryoblastic cell line CHRF-288. *Arterioscler Thromb* 1993; 13: 669–674.
181 Medina R, Socher SH, Han JH, Friedman PA. Interleukin-1, endotoxin or tumor necrosis factor/cachectin enhance the level of plasminogen activator inhibitor messenger RNA in bovine aortic endothelial cells. *Thromb Res* 1989; 54: 41–52.
182 Bevilacqua MP, Schleef RR, Gimbrone MA, Jr., Loskutoff DJ. Regulation of the fibrinolytic system of cultured human vascular endothelium by interleukin 1. *J Clin Invest* 1986; 78: 587–591.
183 Stiko-Rahm A, Wiman B, Hamsten A, Nilsson J. Secretion of plasminogen activator inhibitor-1 from cultured human umbilical vein endothelial cells is induced by very low density lipoprotein. *Arteriosclerosis* 1990; 10: 1067–1073.
184 Olson JA, Jr., Shiverick KT, Ogilvie S, Buhi WC, Raizada MK. Angiotensin II induces secretion of plasminogen activator inhibitor 1 and a tissue metalloprotease inhibitor-related protein from rat brain astrocytes. *Proc Natl Acad Sci USA* 1991; 88: 1928–1932.
185 Grzywacz A, Elikowski W, Psuja P, Zozulinska M, Zawilska K. Impairment of plasma fibrinolysis in young survivors of myocardial infarction with silent ischaemia. *Blood Coagul Fibrinolysis* 1998; 9: 245–249.
186 Sakata K, Kurata C, Kobayashi A, Rydzewski A, Yamazaki N. Plasminogen activator inhibitor activity as a possible indicator of disease activity in rest angina with angiographically insignificant coronary artery stenosis. *Thromb Res* 1991; 63: 491–502.
187 Huber K, Resch I, Stefenelli T et al. Plasminogen activator inhibitor-1 levels in patients with chronic angina pectoris with or without angiographic evidence of coronary sclerosis. *Thromb Haemost* 1990; 63: 336–339.
188 Davi G, Violi F, Catalano I et al. Increased plasminogen activator inhibitor antigen levels in diabetic patients with stable angina. *Blood Coagul Fibrinolysis* 1991; 2: 41–45.
189 Cigolini M, Targher G, Seidell JC et al. Relationships of plasminogen activator inhibitor-1 to anthropometry, serum insulin, triglycerides and adipose tissue fatty acids in healthy men. *Atherosclerosis* 1994; 106: 139–147.
190 Byberg L, Siegbahn A, Berglund L, McKeigue P, Reneland R, Lithell H. Plasminogen activator inhibitor-1 activity is independently related to both insulin sensitivity and serum triglycerides in 70-year-old men. *Arterioscler Thromb Vasc Biol* 1998; 18: 258–264.
191 Hasenstab D, Lea H, Clowes AW. Local plasminogen activator inhibitor type 1 overexpression in rat carotid artery enhances thrombosis and endothelial regeneration while inhibiting intimal thickening. *Arterioscler Thromb Vasc Biol* 2000; 20: 853–859.
192 Kawasaki T, Dewerchin M, Lijnen HR, Vermylen J, Hoylaerts MF. Vascular release of plasminogen activator inhibitor-1 impairs fibrinolysis during acute arterial thrombosis in mice. *Blood* 2000; 96: 153–160.
193 Ren S, Man RY, Angel A, Shen GX. Oxidative modification enhances lipoprotein(a)-induced overproduction of plasminogen activator inhibitor-1 in cultured vascular endothelial cells. *Atherosclerosis* 1997; 128: 1–10.
194 Lollar P, Owen WG. Clearance of thrombin from circulation in rabbits by high-affinity binding sites on endothelium. Possible role in the inactivation of thrombin by antithrombin III. *J Clin Invest* 1980; 66: 1222–1230.
195 Marcum JA, McKenney JB, Rosenberg RD. Acceleration of thrombin-antithrombin complex formation in rat hindquarters via heparinlike molecules bound to the endothelium. *J Clin Invest* 1984; 74: 341–350.
196 Busch C, Owen WG. Identification in vitro of an endothelial cell surface cofactor for antithrombin III. Parallel studies with isolated perfused rat hearts and microcarrier cultures of bovine endothelium. *J Clin Invest* 1982; 69: 726–729.
197 Stern D, Nawroth P, Marcum J et al. Interaction of antithrombin III with bovine aortic segments. Role of heparin in binding and enhanced anticoagulant activity. *J Clin Invest* 1985; 75: 272–279.
198 Oldberg A, Heldin CH, Wasteson A, Busch C, Hook M.

Cambridge Books Online © Cambridge University Press, 2009

Characterization of a platelet endoglycosidase degrading heparin-like polysaccharides. *Biochemistry* 1980; 19: 5755–5762.
199 Walker FJ, Sexton PW, Esmon CT. The inhibition of blood coagulation by activated Protein C through the selective inactivation of activated Factor V. *Biochim Biophys Acta* 1979; 571: 333–342.
200 Marlar RA, Kleiss AJ, Griffin JH. Mechanism of action of human activated protein C, a thrombin-dependent anticoagulant enzyme. *Blood* 1982; 59: 1067–1072.
201 Walker FJ. Regulation of activated protein C by protein S. The role of phospholipid in factor Va inactivation. *J Biol Chem* 1981; 256: 11128–11131.
202 Maruyama I, Majerus PW. The turnover of thrombin-thrombomodulin complex in cultured human umbilical vein endothelial cells and A549 lung cancer cells. Endocytosis and degradation of thrombin. *J Biol Chem* 1985; 260: 15432–15438.
203 Adnot S, Joseph D, Vargaftig BB. Antagonists of PAF-acether do not suppress thrombin-induced aggregation of ADP-deprived and aspirin-treated human platelets. *Agents Actions* 1987; 21: 195–202.
204 Ofosu FA, Hirsh J, Esmon CT et al. Unfractionated heparin inhibits thrombin-catalysed amplification reactions of coagulation more efficiently than those catalysed by factor Xa. *Biochem J* 1989; 257: 143–150.
205 McKee PA, Schwartz ML, Pizzo SV, Hill RL. Cross-linking of fibrin by fibrin-stabilizing factor. *Ann NY Acad Sci* 1972; 202: 127–148.
206 Nakamura K, Hatano Y, Mori K. Thrombin-induced vasoconstriction in isolated cerebral arteries and the influence of a synthetic thrombin inhibitor. *Thromb Res* 1985; 40: 715–720.
207 Hirsh J, Piovella F, Pini M. Congenital antithrombin III deficiency. Incidence and clinical features. *Am J Med* 1989; 87: 34S–38S.
208 Marcum JA, Atha DH, Fritze LM, Nawroth P, Stern D, Rosenberg RD. Cloned bovine aortic endothelial cells synthesize anticoagulantly active heparan sulfate proteoglycan. *J Biol Chem* 1986; 261: 7507–7517.
209 Comp PC, Esmon CT. Recurrent venous thromboembolism in patients with a partial deficiency of protein S. *N Engl J Med* 1984; 311: 1525–1528.
210 Dahlback B. Resistance to activated protein C as risk factor for thrombosis: molecular mechanisms, laboratory investigation, and clinical management. *Semin Hematol* 1997; 34: 217–234.
211 Lentz SR, Sadler JE. Inhibition of thrombomodulin surface expression and protein C activation by the thrombogenic agent homocysteine. *J Clin Invest* 1991; 88: 1906–1914.
212 Broze GJ, Jr., Warren LA, Novotny WF, Higuchi DA, Girard JJ, Miletich JP. The lipoprotein-associated coagulation inhibitor that inhibits the factor VII-tissue factor complex also inhibits factor Xa: insight into its possible mechanism of action. *Blood* 1988; 71: 335–343.
213 Stern DM, Nawroth PP, Kisiel W, Vehar G, Esmon CT. The binding of factor IXa to cultured bovine aortic endothelial cells. Induction of specific site in the presence of factors VIII and X. *J Biol Chem* 1985; 260: 6717–6722.
214 Taubman MB. Tissue factor regulation in vascular smooth muscle: a summary of studies performed using in vivo and in vitro models. *Am J Cardiol* 1993; 72: 55C–60C.
215 Wilcox JN, Smith KM, Schwartz SM, Gordon D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. *Proc Natl Acad Sci USA* 1989; 86: 2839–2843.
216 Brox JH, Osterud B, Bjorklid E, Fenton JWD. Production and availability of thromboplastin in endothelial cells: the effects of thrombin, endotoxin and platelets. *Br J Haematol* 1984; 57: 239–246.
217 Schorer AE, Rick PD, Swaim WR, Moldow CF. Structural features of endotoxin required for stimulation of endothelial cell tissue factor production; exposure of preformed tissue factor after oxidant-mediated endothelial cell injury. *J Lab Clin Med* 1985; 106: 38–42.
218 Yang Y, Loscalzo J. Regulation of tissue factor expression in human microvascular endothelial cells by nitric oxide. *Circulation* 2000; 101: 2144–2148.
219 Seigel G, Schnalke F, Ruckborn K, Hetzer R. Role of prostacyclin in normal and arteriosclerotic human coronary arteries during hypoxia. *Agents Actions Supplement* 1992; 37: 320–323.
220 Tesfamariam B, Brown ML, Deykin D, Cohen RA. Elevated glucose promotes generation of endothelium-derived vasoconstrictor prostanoids in rabbit aorta. *J Clin Invest* 1990; 85: 929–932.
221 Cohen RA. Dysfunction of vascular endothelium in diabetes mellitus. *Circulation* 1993; 87: V67–V76.
222 Taddei S, Virdis A, Mattei P, Salvetti A. Vasodilation to acetylcholine in primary and secondary forms of human hypertension. *Hypertension* 1993; 21: 929–933.
223 Ludmer PL, Selwyn AP, Shook TL et al. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *N Engl J Med* 1986; 315: 1046–1051.
224 Creager MA, Cooke JP, Mendelsohn ME et al. Impaired vasodilation of forearm resistance vessels in hypercholesterolemic humans. *J Clin Invest* 1990; 86: 228–234.
225 Sorensen KE, Celermajer DS, Georgakopoulos D, Hatcher G, Betteridge DJ, Deanfield JE. Impairment of endothelium-dependent dilation is an early event in children with familial hypercholesterolemia and is related to the lipoprotein(a) level. *J Clin Invest* 1994; 93: 50–55.
226 Crossman DC, Larkin SW, Dashwood MR, Davies GJ, Yacoub M, Maseri A. Responses of atherosclerotic human coronary arteries in vivo to the endothelium-dependent vasodilator substance P. *Circulation* 1991; 84: 2001–2010.
227 Cox DA, Vita JA, Treasure CB et al. Atherosclerosis impairs flow-mediated dilation of coronary arteries in humans. *Circulation* 1989; 80: 458–465.
228 Zeiher AM, Drexler H, Wollschlager H, Just H. Modulation of

Cambridge Books Online © Cambridge University Press, 2009

coronary vasomotor tone in humans. Progressive endothelial dysfunction with different early stages of coronary atherosclerosis. *Circulation* 1991; 83: 391–401.

229 Glassman AB. Platelet abnormalities in diabetes mellitus. *Ann Clin Lab Sci* 1993; 23: 47–50.

230 Chirkov YY, Holmes AS, Chirkova LP, Horwitz JD. Nitrate resistance in platelets from patients with stable angina pectoris [see comments]. *Circulation* 1999; 100: 129–134.

231 Freedman JE, Ting B, Hankin B, Loscalzo J, Keaney JF, Jr., Vita JA. Impaired platelet production of nitric oxide predicts presence of acute coronary syndromes. *Circulation* 1998; 98: 1481–1486.

232 Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia increases endothelial superoxide anion production. *J Clin Invest* 1993; 91: 2546–2551.

233 Tesfamariam B, Cohen RA. Free radicals mediate endothelial cell dysfunction caused by elevated glucose. *Am J Physiol* 1992; 263: H321–H326.

234 Rajagopalan S, Kurz S, Munzel T et al. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest* 1996; 97: 1916–1923.

235 Levine GN, Frei B, Koulouris SN, Gerhard MD, Keaney JF, Jr., Vita JA. Ascorbic acid reverses endothelial vasomotor dysfunction in patients with coronary artery disease [see comments]. *Circulation* 1996; 93: 1107–1113.

236 De la Cruz JP, Garcia PJ, Sanchez de la Cuesta F. Dipyridamole inhibits platelet aggregation induced by oxygen-derived free radicals. *Thromb Res* 1992; 66: 277–285.

237 Bordia A, Verma SK. Effect of vitamin C on platelet adhesiveness and platelet aggregation in coronary artery disease patients. *Clin Cardiol* 1985; 8: 552–554.

238 Gryglewski RJ, Palmer RM, Moncada S. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature* 1986; 320: 454–456.

239 Rubanyi GM, Vanhoutte PM. Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. *Am J Physiol* 1986; 250: H822–H827.

240 Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* 1996; 271: C1424–C1437.

241 Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 1990; 87: 1620–1624.

242 Weiss SJ, Young J, LoBuglio AF, Slivka A, Nimeh NF. Role of hydrogen peroxide in neutrophil-mediated destruction of cultured endothelial cells. *J Clin Invest* 1981; 68: 714–721.

243 Wei EP, Kontos HA. H2O2 and endothelium-dependent cerebral arteriolar dilation. Implications for the identity of endothelium-derived relaxing factor generated by acetylcholine. *Hypertension* 1990; 16: 162–169.

244 Cushing SD, Berliner JA, Valente AJ et al. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc Natl Acad Sci USA* 1990; 87: 5134–5138.

245 Cybulsky MI, Gimbrone MA, Jr. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science* 1991; 251: 788–791.

246 McNally AK, Chisolm GMd, Morel DW, Cathcart MK. Activated human monocytes oxidize low-density lipoprotein by a lipoxygenase-dependent pathway. *J Immunol* 1990; 145: 254–259.

247 Negre-Salvayre A, Pieraggi MT, Mabile L, Salvayre R. Protective effect of 17 beta-estradiol against the cytotoxicity of minimally oxidized LDL to cultured bovine aortic endothelial cells. *Atherosclerosis* 1993; 99; 207–217.

248 Liao JK, Shin WS, Lee WY, Clark SL. Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. *J Biol Chem* 1995; 270: 319–324.

249 Kugiyama K, Kerns SA, Morrisett JD, Roberts R, Henry PD. Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. *Nature* 1990; 344: 160–162.

250 Keaney JF, Jr., Guo Y, Cunningham D, Shwaery GT, Xu A, Vita JA. Vascular incorporation of alpha-tocopherol prevents endothelial dysfunction due to oxidized LDL by inhibiting protein kinase C stimulation. *J Clin Invest* 1996; 98: 386–394.

251 Schmidt K, Klatt P, Graier WF, Kostner GM, Kukovetz WR. High-density lipoprotein antagonizes the inhibitory effects of oxidized low-density lipoprotein and lysolecithin on soluble guanylyl cyclase. *Biochem Biophys Res Commun* 1992; 182: 302–308.

252 Harrison DG. Cellular and molecular mechanisms of endothelial cell dysfunction. *J Clin Invest* 1997; 100: 2153–2157.

252a Loscalzo J. Nitric oxide insufficiency, platelet activation and arterial thrombosis. *Circ Res* 2001; 88: 756–762.

253 Robson SC, Kaczmarek E, Siegal JB et al. Loss of ATP diphosphohydrolase activity with endothelial cell activation. *J Exp Med* 1997; 185: 153–163.

254 Forstermann U, Mugge A, Bode SM, Frolich JC. Response of human coronary arteries to aggregating platelets: importance of endothelium-derived relaxing factor and prostanoids. *Circ Res* 1988; 63: 306–312.

255 Vita JA, Treasure CB, Nabel EG et al. Coronary vasomotor response to acetylcholine relates to risk factors for coronary artery disease [see comments]. *Circulation* 1990; 81: 491–497.

256 Crossman DC, Larkin SW, Fuller RW, Davies GJ, Maseri A. Substance P dilates epicardial coronary arteries and increases coronary blood flow in humans. *Circulation* 1989; 80: 475–484.

257 Matsuyama K, Yasue H, Okumura K et al. Effects of H1-receptor stimulation on coronary arterial diameter and coronary hemodynamics in humans. *Circulation* 1990; 81: 65–71.

258 Shimokawa H, Vanhoutte PM. *Endothelium and vascular*

Cambridge Books Online © Cambridge University Press, 2009

injury in hypertension and atherosclerosis. New York: Elsevier Science; 1997.

259 Vanhoutte PM, Shimokawa H. Endothelium-derived relaxing factor and coronary vasospasm. *Circulation* 1989; 80: 1–9.

260 Gordon JB, Ganz P, Nabel EG et al. Atherosclerosis influences the vasomotor response of epicardial coronary arteries to exercise. *J Clin Invest* 1989; 83: 1946–1952.

261 Drexler H, Hornig B. Endothelial dysfunction in human disease. *J Mol Cell Cardiol* 1999; 31: 51–60.

262 Vanhoutte PM. Hypercholesterolaemia, atherosclerosis and release of endothelium-derived relaxing factor by aggregating platelets. *Eur Heart J* 1991; 12: 25–32.

263 Creager MA, Gallagher SJ, Girerd XJ, Coleman SM, Dzau VJ, Cooke JP. L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. *J Clin Invest* 1992; 90: 1248–1253.

264 Vanhoutte PM, Boulanger CM. Endothelium-dependent responses in hypertension. *Hypertens Res* 1995; 18: 87–98.

265 Ge T, Hughes H, Junquero DC, Wu KK, Vanhoutte PM, Boulanger CM. Endothelium-dependent contractions are associated with both augmented expression of prostaglandin H synthase-1 and hypersensitivity to prostaglandin H2 in the SHR aorta. *Circ Res* 1995; 76: 1003–1010.

266 Lüscher TF, Raij L, Vanhoutte PM. Endothelium-dependent vascular responses in normotensive and hypertensive Dahl rats. *Hypertension* 1987; 9: 157–163.

267 Panza JA, Quyyumi AA, Brush JE, Jr., Epstein SE. Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension [see comments]. *N Engl J Med* 1990; 323: 22–27.

268 Panza JA, Casino PR, Badar DM, Quyyumi AA. Effect of increased availability of endothelium-derived nitric oxide precursor on endothelium-dependent vascular relaxation in normal subjects and in patients with essential hypertension [see comments]. *Circulation* 1993; 87: 1475–1481.

269 Tesfamariam B, Jakubowski JA, Cohen RA. Contraction of diabetic rabbit aorta caused by endothelium-derived PGH2-TxA2. *Am J Physiol* 1989; 257: H1327–H1333.

270 Oyama Y, Kawasaki H, Hattori Y, Kanno M. Attenuation of endothelium-dependent relaxation in aorta from diabetic rats. *Eur J Pharmacol* 1986; 132: 75–78.

271 Jorgensen RG, Russo L, Mattioli L, Moore WV. Early detection of vascular dysfunction in type I diabetes. *Diabetes* 1988; 37: 292–296.

272 Clarkson P, Celermajer DS, Donald AE et al. Impaired vascular reactivity in insulin-dependent diabetes mellitus is related to disease duration and low density lipoprotein cholesterol levels. *J Am Coll Cardiol* 1996; 28: 573–579.

273 Arcaro G, Zenere BM, Saggiani F et al. ACE inhibitors improve endothelial function in type 1 diabetic patients with normal arterial pressure and microalbuminuria. *Diabetes Care* 1999; 22: 1536–1542.

274 Enderle MD, Benda N, Schmuelling RM, Haering HU, Pfohl M. Preserved endothelial function in IDDM patients, but not in NIDDM patients, compared with healthy subjects [see comments]. *Diabetes Care* 1998; 21: 271–277.

275 Lambert J, Aarsen M, Donker AJ, Stehouwer CD. Endothelium-dependent and -independent vasodilation of large arteries in normoalbuminuric insulin-dependent diabetes mellitus. *Arterioscler Thromb Vasc Biol* 1996; 16: 705–711.

276 De Vriese AS, Verbeuren TJ, Van de Voorde J, Lamiere NH, Vanhoutte PM. Endothelial dysfunction in diabetes. *Br J Pharmacol* 2000; 130: 963–974.

277 Clarke R, Daly L, Robinson K et al. Hyperhomocysteinemia: an independent risk factor for vascular disease [see comments]. *N Engl J Med* 1991; 324: 1149–1155.

278 Brattstrom LE, Hardebo JE, Hultberg BL. Moderate homocysteinemia – a possible risk factor for arteriosclerotic cerebrovascular disease. *Stroke* 1984; 15: 1012–1016.

279 Stamler JS, Osborne JA, Jaraki O et al. Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. *J Clin Invest* 1993; 91: 308–318.

280 Wall RT, Harlan JM, Harker LA, Striker GE. Homocysteine-induced endothelial cell injury in vitro: a model for the study of vascular injury. *Thromb Res* 1980; 18: 113–121.

281 Lentz SR, Sobey CG, Piegors DJ et al. Vascular dysfunction in monkeys with diet-induced hyperhomocyst(e)inemia [see comments]. *J Clin Invest* 1996; 98: 24–29.

282 Celermajer DS, Sorensen K, Ryalls M et al. Impaired endothelial function occurs in the systemic arteries of children with homozygous homocystinuria but not in their heterozygous parents. *J Am Coll Cardiol* 1993; 22: 854–858.

283 Woo KS, Chook P, Lolin YI et al. Hyperhomocyst(e)inemia is a risk factor for arterial endothelial dysfunction in humans [published erratum apperas in *Circulation* 2000 Mar 28; 101: E116]. *Circulation* 1997; 96: 2542–2544.

284 Usui M, Matsuoka H, Miyazaki H, Ueda S, Okuda S, Imaizumi T. Endothelial dysfunction by acute hyperhomocyst(e)inaemia: restoration by folic acid. *Clin Sci (Colch)* 1999; 96: 235–239.

285 Tawakol A, Omland T, Gerhard M, Wu JT, Creager MA. Hyperhomocyst(e)inemia is associated with impaired endothelium-dependent vasodilation in humans [published erratum appears in *Circulation* 2000 Mar 28; 101: E116]. *Circulation* 1997; 95: 1119–1121.

286 Starkebaum G, Harlan JM. Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. *J Clin Invest* 1986; 77: 1370–1376.

287 Upchurch GR, Jr., Welch GN, Fabian AJ, Pigazzi A, Keaney JF, Jr., Loscalzo J. Stimulation of endothelial nitric oxide production by homocyst(e)ine. *Atherosclerosis* 1997; 132: 177–185.

288 Upchurch GR, Jr., Welch GN, Fabian AJ. Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. *J Biol Chem* 1997; 262: 17012–17017.

Cambridge Books Online © Cambridge University Press, 2009

289 Di Minno G, Coppola A, Mancini FP, Margaglione M. Homocysteine, platelet function and thrombosis. *Haematologica* 1999; 84: 61–63.
290 Coppola A, Davi G, De Stafano V, Mancini FP, Cerbone AM, Di Minno G. Homocysteine, coagulation, platelet function, and thrombosis [In Process Citation]. *Semin Thromb Hemost* 2000; 26: 243–254.
291 Di Minno G, Davi G, Margaglione M et al. Abnormally high thromboxane biosynthesis in homozygous homocystinuria. Evidence for platelet involvement and probucol-sensitive mechanism. *J Clin Invest* 1993; 92: 1400–1406.
292 Folts JD, Rowe GG. Cyclic reductions in coronary blood flow in coronary arteries with fixed partial obstruction and their inhibition by aspirin. *Fed Proc* 1974; 33: 413.
293 Folts J. An in vivo model of experimental arterial stenosis, intimal damage, and periodic thrombosis. *Circulation* 1991; 83: IV3–IV14.
294 Willerson JT, Golino P, Eidt J, Campbell WB, Buja LM. Specific platelet mediators and unstable coronary artery lesions. Experimental evidence and potential clinical implications. *Circulation* 1989; 80: 198–205.
295 Maalej N, Folts JD. Increased shear stress overcomes the antithrombotic platelet inhibitory effect of aspirin in stenosed dog coronary arteries [see comments]. *Circulation* 1996; 93: 1201–1205.
296 Rovin JD, Stamler JS, Loscalzo J, Folts JD. Sodium nitroprusside, an endothelium-derived relaxing factor congener, increases platelet cyclic GMP levels and inhibits epinephrine-exacerbated in vivo platelet thrombus formation in stenosed canine coronary arteries. *J Cardiovasc Pharmacol* 1993; 22: 626–631.
297 Folts JD, Stamler J, Loscalzo J. Intravenous nitroglycerin infusion inhibits cyclic blood flow responses caused by periodic platelet thrombus formation in stenosed canine coronary arteries. *Circulation* 1991; 83: 2122–2127.
298 Eidt JF, Ashton J, Golino P, McNatt J, Buja LM, Willerson JT. Treadmill exercise promotes cyclic alterations in coronary blood flow in dogs with coronary artery stenoses and endothelial injury. *J Clin Invest* 1989; 84: 517–527.
299 Coller BS, Folts JD, Scudder LE, Smith SR. Antithrombotic effect of a monoclonal antibody to the platelet glycoprotein IIb/IIIa receptor in an experimental animal model. *Blood* 1986; 68: 783–786.
300 Coller BS, Folts JD, Smith SR, Scudder LE, Jordan R. Abolition of in vivo platelet thrombus formation in primates with monoclonal antibodies to the platelet GPIIb/IIIa receptor. Correlation with bleeding time, platelet aggregation, and blockade of GPIIb/IIIa receptors [see comments]. *Circulation* 1989; 80: 1766–1774.
301 Eichhorn EJ, Grayburn PA, Anderson HV, Bedotto JB, Carry MM, Willerson JT. Cyclic coronary artery flow variations before and after percutaneous transluminal coronary angioplasty in man. *Circulation* 1989; 80: II–371.
302 Anderson HV, Willerson JT. Experimental models of thrombosis and thrombolysis. In: Loscalzo J, Schafer AI, eds. *Thrombosis and hemorrhage.* 2nd edn. Baitimore: Williams and Wilkins; 1998; 425–434.
303 Kordenat RK, Kezdi P, Stanley EL. A new catheter technique for producing experimental coronary thrombosis and selective coronary visualization. *Am Heart J* 1972; 83: 360–364.
304 Bergmann SR, Fox KA, Ter-Pogossian MM, Sobel BE, Collen D. Clot-selective coronary thrombolysis with tissue-type plasminogen activator. *Science* 1983; 220: 1181–1183.
305 Cercek B, Lew AS, Hod H, Yano J, Reddy NK, Ganz W. Enhancement of thrombolysis with tissue-type plasminogen activator by pretreatment with heparin. *Circulation* 1986; 74: 583–587.
306 Mahley RW. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science* 1988; 240: 622–630.
307 Zhang SH, Reddick RL, Piedrahita JA, Maeda N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 1992; 258: 468–471.
308 Breslow JL. Transgenic mouse models of lipoprotein metabolism and atherosclerosis. *Proc Natl Acad Sci USA* 1993; 90: 8314–8318.
309 Reddick RL, Zhang SH, Maeda N. Atherosclerosis in mice lacking apo E. Evaluation of lesional development and progression [published erratum appears in *Arterioscler Thromb* 1994 May; 14: 839]. *Arterioscler Thromb* 1994; 14: 141–147.
310 Yang R, Powell-Braxton L, Ogaoawara AK et al. Hypertension and endothelial dysfunction in apolipoprotein E knockout mice. *Arterioscler Thromb Vasc Biol* 1999; 19: 2762–2768.
311 Ohashi Y, Kawashima S, Hirata K et al. Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase [see comments]. *J Clin Invest* 1998; 102: 2061–2071.
312 Huang PL, Huang Z, Mashimo H et al. Hypertension in mice lacking the gene for endothelial nitric oxide synthase [see comments]. *Nature* 1995; 377: 239–242.
313 Watanabe M, Osada J, Aratani Y et al. Mice deficient in cystathionine beta-synthase: animal models for mild and severe homocyst(e)inemia. *Proc Natl Acad Sci USA* 1995; 92: 1585–1589.
314 Eberhardt RT, Forgione MA, Cap A et al. Endothelial dysfunction in a murine model of mild hyperhomocyst(e)inemia. *J Clin Invest* 2000; 106: 483–491.
315 Ursini F, Maiorino M, Brigelius-Flohe R et al. Diversity of glutathione peroxidases. *Methods Enzymol* 1995; 252: 38–53.
316 Esposito LA, Kokoszka JE, Waymire KG, Cottrell B, MacGregor GR, Wallace DC. Mitochondrial oxidative stress in mice lacking the glutathione peroxidase-1 gene. *Free Radic Biol Med* 2000; 28: 754–766.
317 Forgione MA, Heydrick SJ, Trolliet MR et al. Vascular oxidant

Cambridge Books Online © Cambridge University Press, 2009

stress in cystathionine β synthase and glutathione peroxidase deficiency (abst). *Circulation* 1999; 100: I–235.
318 Forgione MA, Heydrick SJ, Cap AP, Weiss N, Loscalzo J. Cellular glutathione peroxidase deficiency and vascular oxidant stress (abst). *Circulation* 2000; 102: II–167.
319 Chen LY, Mehta JL. Further evidence of the presence of constitutive and inducible nitric oxide synthase isoforms in human platelets. *J Cardiovasc Pharmacol* 1996; 27: 154–158.
320 Bodzenta-Lukaszyk A, Gabryelewicz A, Lukaszyk A, Bielawiec M, Konturek JW, Domschke W. Nitric oxide synthase inhibition and platelet function. *Thromb Res* 1994; 75: 667–672.

Cambridge Books Online © Cambridge University Press, 2009

Cambridge Books Online © Cambridge University Press, 2009

Part II

Methodology

Cambridge Books Online © Cambridge University Press, 2009

Cambridge Books Online © Cambridge University Press, 2009

30

In vitro assays for evaluating platelet function

Perumal Thiagarajan and Kenneth K. Wu

Vascular Biology Research Center and Division of Hematology, University of Texas Medical School, Houston, USA

Role of platelets in hemostasis and thrombosis

Platelets play a primary role in the formation of the hemostatic plug at sites of vessel wall injury and in formation of thrombotic plug at the sites of atherosclerotic lesions[1–3]. Platelets circulate as anucleated cells at the periphery of the bloodstream. When continuity of the endothelial layer is disrupted and the underlying matrix is exposed, platelets rapidly adhere to the exposed matrix through an interaction between the glycoprotein (Gp) Ib–IX–V complex on their surface and von Willebrand factor (vWf) in the subendothelium[2,3]. The high fluid shear stress brought about by vascular constriction also promotes Gp Ib–IX–V–von Willebrand factor interaction[3]. This initial interaction, often called platelet adhesion, sets the stage for other adhesive reactions that allow the platelets to essentially seal the vessel-wall defect.

Following adhesion, platelets are activated by a number of agonists such as adenosine diphosphate (ADP) and collagen present at the sites of vascular injury. These agonists activate platelets by binding to specific receptors on the platelet surface[4]. Occupancy of these receptors leads to a series of downstream events that ultimately increases the intracytoplasmic concentration of calcium ions[5]. Receptors coupled to the G- proteins such as ADP, epinephrine, thromboxane A2 (TXA2) and thrombin receptors activate phospholipase Cβ (PLCb), whereas receptors activating via the non-receptor tyrosine kinase pathways such as collagen receptor GpVI preferentially activate phospholipase Cγ (PLCγ)[6]. Activation of PLCβ or PLCγ results in the production of the two second messengers: diacylglycerol (DAG) and inositol trisphosphate (IP3). DAG activates protein kinase C while the IP3 liberates calcium from intracellular storage granules. In addition, some of the receptors, such as ADP and TXA2 receptors, may also be linked via the G-proteins to ion channels mediating influx of Ca^{2+}. The increased intraplatelet free calcium concentration, in turn, leads to a number of structural and functional changes of the platelet. Morphologically, the platelet changes dramatically from a disc to a spiny sphere (a process called shape change). The granules in the platelet are centralized and their contents (ADP, β-thromboglobulin, platelet factor 4) are discharged into the lumen of the open canalicular system, from which they are then released to the exterior (the release reaction). The increase in intraplatelet calcium stimulates membrane phospholipase A2 activity, which liberates arachidonic acid from membrane phospholipids. Arachidonic acid is converted to an intermediate product prostaglandin H2 (PGH2) by the enzyme cyclooxygenase 1 (COX-1). PGH2 is further metabolized to TXA2 by thromboxane synthase. TXA2 is a very potent activator of platelets. The long membrane projections brought about by a shape-change reaction allow the platelets to interact with one another to form aggregates.

The platelet-platelet interaction (platelet aggregation) is mediated by a membrane protein, GPIIb–IIIa complex[7]. GPIIb–IIIa is an integrin receptor present at high density on platelets (~50 000 copies/platelet). It exists as an inactive form in resting platelets. Platelet activation by almost all agonists induces conformational changes (‘inside-out signalling’) of GPIIb–IIIa which becomes competent to bind soluble plasma fibrinogen. The precise sequence of events leading to inside-out signalling has not been fully elucidated. The roles of receptor clustering, phosphorylation and association with cytoskeletal and other cytoplasmic molecules in inducing GPIIb–IIIa conformational change leading to ligand binding are not totally delineated. Nevertheless, the receptor-bound fibrinogen acts as a bridge between two GPIIb–IIIa on adjacent platelets. This is the final common pathway of platelet aggregation induced by platelet chemical agonists. However, vWF substitutes for fibrinogen as a bridge molecule between

GPIIb–IIIa for platelet aggregation induced by high shear stresses ($>$12 dynes/cm^2) although platelet aggregation under lower shear stresses ($<$12 dynes/cm^2) is mediated by fibrinogen binding to GPIIb–IIIa[7–9].

Activated platelets recruit additional platelets to the growing hemostatic plug by several feedback amplification loops: they release platelet agonists (such as ADP and seratonin stored in the α granules) and they synthesize *de novo* proaggregatory TXA_2. Release of ADP and TXA_2 synthesis consolidate the initial hemostatic plug by promoting the participation of other platelets in the hemostatic plug formed at sites of vascular injury. Finally, platelets also play a dominant role in secondary hemostasis by providing a highly effective catalytic surface for activation of the coagulation cascade. When platelets are activated, negatively charged phospholipids move from the inner leaflet of the membrane bilayer to the outer leaflet. The transbilayer movement of anionic phospholipids is associated with blebbing and release of procoagulant vesicles rich in anionic phospholipids. Both activated platelets and the microvesicles provide binding sites for enzymes and cofactors of the coagulation system, which then efficiently generate thrombin, itself a potent platelet agonist.

Thrombosis results from inappropriate activation of normal hemostatic process in the setting of vessel wall injury. Arterial thrombosis occurs at high flow rates and at sites of atherosclerotic lesions. Clinical, experimental and epidemiological studies have clearly demonstrated a dominant role for platelets in arterial thrombosis. Platelets adhere to the exposed matrix of ruptured atheromatous plaque by a biochemical mechanism similar to that described above for adhesion of platelets to subendothelial matrix of severed normal vessels. Subsequent events such as platelet activation, platelet aggregation and platelet participation in coagulation are also similar to those described above. A notable pathophysiological process in arterial thrombosis is platelet aggregation induced by high shear stress which is mediated by binding of vWF to GPIIb–IIIa[7–9]. This contributes to the initiation and amplification of platelet aggregates not generally noted in normal hemostasis.

Platelet function tests

There have been attempts over the past three decades to detect changes in platelet function and correlate the change with arterial thrombotic events in patients with arterial thrombotic disorders[10]. There have been studies on the association of platelet function and venous thromboembolism, which is described in detail in another chapter in this book. The goal of the platelet function testing is to detect early thrombotic events and even more importantly to predict risk for thrombotic disorders such as myocardial infarction and ischemic stroke. There is circumstantial evidence for individual and interethnic variations of platelet function which forms the basic assumption that interindividual variability of platelet function can impact on arterial thrombus formation. This has been a controversial subject. However, recent genetic analysis of platelet glycoprotein genes suggests that polymorphisms of these genes contribute to variations in the receptor affinity and the magnitude of transmembrane signalling and these detectable interindividual differences may translate to different risk of arterial thrombosis. Environmental factors such as dietary cholesterol, cigarette smoking and exercise influence platelet function[11]. Furthermore, there could be genetic and environmental interactions in influencing platelet function in healthy individuals that collectively may have a larger impact on the risk of arterial thrombosis. Each of these factors contribute to a small variation of platelet function. Therefore, it is highly desirable to have tests that measure small changes in platelet function to assess the risk of thrombosis. Our understanding of mechanisms of platelet-vessel wall interactions has increased rapidly in the last decade. This has led to the introduction of new and potent antiplatelet drugs to treat thrombotic disorders[12]. Many of these drugs are associated with severe hemorrhagic complications. Thus, there is also a need to develop simple reliable platelet function tests that mimic in vivo platelet activity and can guide appropriate dosing of the new and powerful antiplatelet therapy. The platelet function tests, developed over the last four decades to test platelet function, are suited to detect platelet functional defects in bleeding disorders but are limited in capacity to assess platelet hyperactivity in thrombosis. A combined genetic and biochemical analysis of markers may alleviate the limitations. In this chapter we will review platelet function tests with emphasis on recently developed tests.

Tests of global platelet functions

Bleeding time

Bleeding time is the most familiar test of global platelet function. It has been in practice for a century and has been modified several times[13]. In this test, a blood pressure cuff is inflated on the upper arm to a pressure of 40 mm of Hg and a disposable, automated device inflicts a standardized cut of 10 mm length and 1 mm in depth on the volar surface of the forearm. The wound is gently blotted every 30 seconds until bleeding stops. The normal bleeding time is

Cambridge Books Online © Cambridge University Press, 2009

less than 10 minutes. The arrest of bleeding from this superficial wound is almost exclusively due to platelet adhesion and aggregation. As there is progressive prolongation of bleeding time when platelet counts are less than 100 000 per microlitre, the bleeding time test has limited value to detect functional platelet defects in thombocytopenic patients[14]. A prolonged bleeding time in the presence of normal platelet count is very significant indicating a qualitative dysfunction of platelets.

Despite its simplicity, the clinical utility of the bleeding time test has been called into question[15]. It is difficult to perform the test with consistency. It is greatly influenced by the skill and experience of the operator. The depth of the wound, local skin thickness, skin temperature and vascular patterns affect the results. A critical review of bleeding time concluded that bleeding time is not a specific indicator of platelet function and a poor indicator of bleeding risk[15]. A shortened bleeding time was claimed to be associated with arterial thrombosis but the claim has not been validated by rigorous proof.

Several attempts have been made to measure global platelet functions in primary hemostasis by reliable, simple, reproducible tests in vitro under flow conditions. Two new tests have been introduced and evaluated in clinical laboratories for their utility in detecting platelet functional defect in primary hemostasis. They have the potential to replace the conventional bleeding time technique. These two tests, the PFA-100 in vitro platelet function analyser and the Xylum clot signature analyser are described below.

PFA-100 in vitro platelet function analyser

This is a bench-top automated instrument based on an original principle described by Kratzer et al. that assess primary hemostasis under shear stress[16]. It uses a disposable test cartridge which contains a cup and a nitrocellulose membrane at the bottom with a central aperture of 150 μm in diameter. The nitrocellulose membrane is impregnated with type I equine fibrillar collagen plus ADP (Col/ADP membrane) or epinephrine (Col/Epi membrane). The cartridge is inserted into the instrument and kept at 37 °C. A blood sample of 0.8 ml of citrated blood is placed in a cup and aspirated at 200–240 dynes/cm^2 through the aperture. The shear stress and the agonists in the membrane activate platelets leading to platelet aggregation. The end point expressed as closure time is the stopping of blood flow due to occlusion of aperture by platelet aggregates. The platelet aggregate formation depends on (i) vWF binding to collagen-coated nitrocellulose membranes, (ii) platelet adhesion to vWF via platelet glycoprotein Ib–IX–V complex, (iii) platelet activation and (iv) platelet aggregation mediated by interaction of GPIIb–IIIa with vWF and fibrinogen. Normal closure times range from 77–133 seconds for Col/ADP membrane and 98–185 seconds for Col/Epi membrane. This instrument has been tested in bleeding disorders but has not been evaluated in thrombotic disorders. The closure time using the collagen/epinephrine (CEPI) cartridge is abnormal in patients with congenital platelet function defects, von Willebrand disease and aspirin ingestion while the closure time with collagen/ADP (CADP) cartridge is abnormal mainly in patients with von Willebrand disease or thrombocytopathies[17–20]. Aspirin prolongs the closure time 94% of the time with CEPI cartridge and only 27% of the time with CADP cartridge. A dose-dependent prolongation of Col/Epi closure time by aspirin is recently reported[20]. Glanzmann's thrombasthenia, Bernard–Soulier syndrome and most mild von Willebrand disease have a prolonged closure time with both cartridges, while storage pool defect and giant platelet thrombopathy have a prolonged closure time with CEPI cartridge only[18]. The advantages of this instrument include simplicity and reproducibility. It has been reported to have a coefficient of variation of less than 10%. It may have a use in determining the association of global platelet function with arterial thrombosis and in assessing the efficacy and complication of antiplatelet therapy.

Clot signature analyser

The Xylum clot signature analyzer (CSA) is an automated test of primary hemostasis which provides information about platelet adhesion, platelet aggregation and coagulation reactions in a more physiological milieu without adding platelet agonists[21–23]. Fresh blood is perfused, under a microprocessor control, in two separate channels (punch and collagen channel) under constant flow conditions simulating physiological blood flow. The luminal pressure is measured continuously. In the punch channel, vascular injury is simulated by piercing the blood line with a needle causing a sudden decrease in the pressure. The punch hole closes as platelets aggregate and form a hemostatic plug. The time from the punch to recovery of the pressure due to the formation of the hemostaic plug is reported as platelet hemostasis time. The blood flowing in the lumen eventually coagulates which stops the blood flow and causes the pressure to drop to 0. The time interval is reported as the clotting time. In the collagen channel, type I bovine collagen (1.9 cm long) is placed on the lumen aligned to the direction of the blood flow. As blood flows, platelets adhere to collagen leading to thrombus formation and lumen occlusion causing a continuing drop of blood pressure eventually to zero. The time interval from the start of blood flow to 50% drop in luminal pressure is reported

Cambridge Books Online © Cambridge University Press, 2009

as collagen-induced thrombus formation. The CSA mimics physiological conditions better than any other instrument. It is currently undergoing clinical evaluation in a number of conditions. Its utility as predictor of arterial thrombosis remains to be determined.

Platelet aggregation tests

A myriad of methods have been developed for testing platelet aggregation over the last four decades. These tests are classified into the following categories: platelet aggregation tests by aggregometer; shear induced platelet aggregation; and platelet aggregation under flow. These tests are considered separately.

Tests for platelet aggregation with aggregometers

Gus Born originally developed a device to measure platelet aggregation based on changes in light transmission of platelet rich plasma (PRP)[24]. This method has been used in detecting reduced platelet aggregation in hereditary and acquired bleeding disorders, bleeding due to drug complication, evaluating new drugs and determining hyperactive platelets in patients with thrombosis. Born's aggregometer was initially developed for assessing platelet function using PRP. Subsequently, aggregometers were developed for assessing platelet function using whole blood[25].

For measuring PRP platelet aggregation, blood is collected by a clean venipuncture in 1/10 volume of citrate (0.109–0.129 M sodium citrate). The PRP is obtained by centrifugation at 150–200 *g* for 10–15 minutes. The PRP is stirred at 37 °C by a magnetic stirrer in an aggregometer with or without addition of an agonist. Light passing through the PRP suspension is measured by a photometer, over a 5–10 minutes period. Kinetics of aggregation as determined photometrically manifests multiple phases. Following the addition of an agonist, there is a change in the shape of platelets from discoid to spherical. This is reflected as a slight decrease in light transmission. This is followed by an increase in light transmission as aggregates are formed. This initial phase of platelet aggregation (primary wave) is reversible unless it is followed by secretion of contents from α and dense granules, which induce an irreversible aggregation (secondary wave). The magnitude of aggregation is detected by measuring the maximum change in light transmission. Some workers have proposed to measure the rate of aggregation (the slope of the curve, i.e. tangent to the curve as the magnitude of aggregation)[26]. These two measurements correlate well[27]. For optimal platelet aggregation in the PRP, platelets come in close contact with one another by stirring. Platelet aggregation requires divalent cations and fibrinogen. Citrated plasmas contain ~40 μM calcium and plasma fibrinogen, which are adequate for the aggregation test.

Spontaneous platelet aggregation

PRP obtained from some individuals form platelet aggregates by stirring alone without stimulation by an agonist. This phenomenon is called spontaneous platelet aggregation (SPA)[28]. SPA was reported to be more frequent in patients with myocardial infarction and ischemic stroke[29]. A prospective study has shown that individuals with SPA have a higher risk of developing myocardial infarction[30]. SPA may represent extremely hyperactive platelets but the exact mechanism by which this occurs has not been elucidated.

Agonist-induced platelet aggregation

ADP, at low concentrations, 0.2–2 μM, causes a biphasic platelet aggregation. The second phase of aggregation induced by low doses of ADP requires TXA_2 synthesis. Aspirin and other cyclooxygenase inhibitors abolish the secondary wave. However, ADP at high concentrations causes a merger of these two phases of aggregation into a single aggregation curve. Epinephrine induces biphasic platelet aggregation at 0.5–7 μM concentration. It does not cause platelet shape change or marked primary aggregation. Thrombin is a potent aggregating agent. However, PRP is unsuitable for thrombin-induced platelet aggregation because thrombin induces blood clotting which alters light transmission. Collagen fibrils do not induce primary aggregation, but platelet adhesion to collagen particles induces secretion of endogenous proaggregatory substances. Collagen-induced platelet aggregation is thus secretion-dependent and very sensitive to aspirin. Arachidonic acid causes platelet aggregation which depends entirely on its transformation to TXA_2. Arachidonic acid-induced platelet aggregation is absent in the presence of cyclooxygenase inhibitors.

Agonist-induced platelet aggregation has been extensively evaluated in patients with myocardial infarction and ischemic stroke[31,32]. In order to provide a more quantitative evaluation of platelet aggregability in thrombosis, a 'threshold' concentration end point has been used[33]. In this assay system platelet aggregation in response to an agonist is titrated using increasing concentrations of the agonist and the minimal concentration of the agonist that induces maximal aggregation is defined as the threshold concentration. Patients with arterial thrombosis have a lower threshold value of ADP, arachidonic acid and collagen-induced platelet aggregation. Taken together, regardless of the end points of aggregation used (threshold concentration, maximal aggregation with a standard con-

Cambridge Books Online © Cambridge University Press, 2009

centration and rate of aggregation) the reported results are consistent with increased platelet aggregability in patients with arterial thrombosis. However, a causal relationship between platelet hyperaggregability and thrombotic events has not been established. The mechanism by which platelet hyperaggregability occurs is also unknown. As mentioned above, there is circumstantial evidence to support the notion that platelet hyperaggregability exists preceding thrombotic events, which may be due to combined genetic and environmental factors.

Ristocetin-induced platelet agglutination

The interaction between vWF and Gp Ib–IX–V complex in vivo following shear stress induces activation of platelets[2,3]. In vitro, the binding of soluble vWF to the GP Ib–IX–V complex under static conditions is artificially induced by modulators such as ristocetin. Ristocetin, a vancomycin-like antibiotic from *Nocardia lurida*, binds to the proline-rich sequence of vWF, altering vWF conformation to facilitate its binding to Gp Ib–IX–V complex. This interaction which does not require divalent cations is called platelet agglutination rather than aggregation. Ristocetin-induced platelet agglutination is defective in von Willebrand disease and in Bernard–Soulier syndrome[34]. In type IIB and pseudo von Willebrand disease there is heightened response to ristocetin[35]. There is limited information regarding changes in ristocetin-induced platelet agglutination in arterial thrombosis.

Platelet aggregometry has several limitations that preclude its routine use in assessing risk of arterial thrombosis. It requires high technical competency and needs standardization in each laboratory at every step of the test, including blood collection, centrifugation speed for the preparation of PRP, adjusting the platelet count and selection of agonist concentration. Furthermore, blood samples need to be processed and tested immediately after blood collection. Furthermore, the test has considerable day-to-day and diurnal variations. However, under stringent conditions, it is a valuable test for determining genetic influence of platelet hyperactivity.

Whole blood platelet aggregation

The whole blood aggregometer eliminates the variabilities associated with preparation of PRP and can be performed in lipemic blood[25,36]. This test is based on measuring the electrical current passed through whole blood where two electrodes are immersed. When the current is passed, the electrodes are immediately coated with a single layer of platelets. The build-up of platelet aggregates impedes the current flow. If no agonist is added there is only a basal accumulation of platelets on the electrodes and the conductance between the electrodes is constant. Following addition of an aggregating agent, platelet aggregates form on the layer of platelets adhered to the electrodes and impair the conduction. This agonist-induced increase in impedance is displayed as a function of time on the recorder.

Several studies have compared platelet aggregation in whole blood to that in PRP and have reported similar results. The value of whole blood aggregometer in assessing platelet function in arterial thrombosis has not been extensively evaluated[37].

Rapid platelet function assay

A new platelet function testing system also using whole blood, the so-called ‘rapid platelet function assay’ (RPFA), has been developed commercially to measure the degree of platelet GPIIb–IIIa blockade[38]. It is an automated turbidometric bead agglutination assay in whole blood, based on the ability of activated platelets to bind fibrinogen. Fibrinogen coated microparticles are mixed with whole blood and the extent of microparticle agglutination is proportional to the quantity of unblocked GPIIb–IIIa on platelets. As activated platelets bind and agglutinate fibrinogen-coated beads, there is an increase in light transmittance. The RPFA device consists of a cartridge and a microprocessor with a digital readout. Results of this assay system correlate very well with the platelet aggregation test performed in PRP with an aggregometer.

Measurement of shear-induced platelet aggregation

Platelet aggregation in response to high shear stress plays a critical role in initiating platelet aggregation at sites of atherosclerotic narrowing of the arteries. High shear stress induces changes in Gp Ib–IX–V complex allowing it to bind vWF multimers and this association accentuates intracellular signalling and GPIIb–IIIa activation. ADP released from platelets and red blood cells is also necessary for shear-induced platelet aggregation[7–9,39].

Shear-induced aggregation in vitro is tested in a rotational viscometer. The cone-and-plate viscometer is the most commonly used rotational viscometer. It generates a constant and uniform shear rate throughout the liquid in between the cone and plate. The shear rate is proportional to the rotations per minute and inversely proportional to the gap angle between the cone and plate. Shear-induced aggregation is quantified by measuring the disappearance of single platelets by electronic counting or more recently by flow cytometry. Several modifications have been made in the instrument to allow real-time optimal measurement of platelet aggregation or calcium flux. Shear-induced aggregation can be performed in whole blood or PRP anticoagulated with heparin or citrate.

Cambridge Books Online © Cambridge University Press, 2009

Shear-induced aggregation is enhanced in myocardial infarction[40]. Shear-induced platelet aggregation is also enhanced in patients with acute stroke and increased aggregation persists 3 months after an acute episode[41]. These results suggest that platelets are more reactive to shear stress in certain subjects which increases their risk of cerebrovascular thrombosis. Prospective studies are needed to determine whether increased shear-induced aggregation is an independent risk factor of arterial thrombosis.

Evaluation of platelet functions under flow

To assess the effect of rheological parameters on the sequence of events leading to formation of mural thrombus resembling physiological conditions, several approaches have been developed[42]. These approaches are based on a similar principle in which platelets were perfused through an appropriate surface under a defined shear rate in a flow chamber in which laminar flow, mimicking in vivo flow conditions is generated. The flow chamber is an annular or parallel plate. The interaction of platelets with the surface components is determined by morphological techniques. Platelets that adhere to the surface are called contact or spread platelets based on their morphological appearance. The subsequent platelet–platelet interaction with the eventual formation of detectable platelet aggregates is called mural platelet thrombus. Several test surfaces including the subendothelium of vessel fragments, cultured vascular cells, artificial surfaces coated with various adhesive molecules (e.g. collagen) have been studied in systems using native anticoagulated blood, citrated PRP or reconstituted blood. In recent years, investigators have combined parallel flow perfusion chambers with computerized epifluorescence microscopy[43]. These techniques, allowing three-dimensional reconstruction of mural thrombus formation in real time have provided valuable information on the role of individual components of platelet activation. Unfortunately, in assessing thrombus formation, these techniques are still within the realm of research laboratories and not suitable for clinical assessment.

Measurements of soluble platelet activation markers

β-thromboglobulin and platelet factor 4

Platelet activation by myriad physiological and pathological stimuli leads to a rapid cytoskeletal contraction, widening of the open canalicular system with eventual secretion of substances stored in α granules, dense bodies and other intracellular granules to the extracellular milieu. These released molecules, such as ADP, play an important role in platelet aggregation and platelet interaction with leukocytes. The released molecules may also serve as a marker of platelet activation *in vivo* because of sensitive assays available for accurately measuring their levels in blood and other body fluids and, more importantly, because of their platelet specificity. β-thromboglobulin (βTG) and platelet factor 4 (PF4) are two such molecules whose utility as a marker of platelet activation in human thrombotic disorders has been extensively investigated[44]. βTG and PF4 are synthesized in megakaryocytes and stored in α-granules of mature platelets. Since they are platelet specific and are secreted into extracellular milieu only after platelet activation, their plasma levels are considered to reflect the extent of platelet activation in vivo. This concept was initially supported by experimental results from in vivo studies[45]. Subsequently, numerous studies show that their levels are elevated in patients with arterial thrombotic diseases such as acute myocardial infarction and thrombotic stroke[46]. However, comparison of plasma βTG or PF4 levels among these studies reveals a large variability in healthy control subjects as well as in patients[47]. It was recognized that a major source of variability is originated from traumatic venipuncture which causes platelet activation and release of βTG and PF4. This source of artificial βTG and PF4 values has been substantially reduced after the introduction of less traumatic venipuncture systems in conjunction with the use of combination anticoagulants consisting of potent antiplatelet and antithrombin reagents[47]. PF4 binds to glycosaminoglycan on endothelial cell surface and has a short half-life in circulating blood. It is less reliable than βTG as a marker of platelet activation. βTG is excreted by kidneys and measurement of urinary βTG levels is considered a reliable test, which is not subject to in vitro platelet activation during venipuncture and blood processing. Plasma βTG levels are elevated in patients with acute myocardial infarction and thrombotic stroke and it has been reported to be associated with subclinical carotid atherosclerosis[48,49]. The value of βTG levels in predicting coronary heart disease and cerebrovascular disease has not been established by prospective studies.

Urinary TXA_2 metabolites

Platelet activation is accompanied by synthesis of TXA_2, which is released at sites of platelet activation. TXA_2 has a very short half-life and is rapidly converted to its stable inactive metabolite TXB_2. TXB_2 is further metabolized to 2,3-dinor-thromboxane B_2 and 11-dehydrothromboxane B_2 and excreted in the urine[50]. The concentration of 11-dehydrothromboxane B_2 in plasma is too low to measure reliably. However, urine 11-dehydrothromboxane B_2 has been determined by radioimmunoassay or enzyme immunoassay. Measurement of 2,3 dinor thromboxane B_2 is also

Cambridge Books Online © Cambridge University Press, 2009

useful, but requires gas chromatography combined with mass spectrometry, which limits its widespread use. 11-dehydrothromboxane B_2 in urine has been shown to be a useful marker of platelet activation and correlates well with βTG levels[51]. However, it should be noted that TXA_2 is also synthesized by activated monocytes and therefore may not be a monospecific marker of platelet activation[52].

Soluble P-selectin

P-selectin is localized to α-granule of platelets and Weibel-Palade body of endothelial cells[53]. A soluble form of P-selectin (sP-selectin) is detected in plasma of healthy subjects, which is thought to originate from an alternatively spliced form of P-selectin[54]. Platelet activation leads to secretion of P-selectin to platelet outer surface and the membrane P-selectin is cleaved by proteolytic enzyme to form sP-selectin[54]. Detection of membrane P-selectin by flow cytometry is accepted as a sensitive method for detecting platelet activation. This will be described in more detail below. Plasma sP-selectin in plasma is also considered as a marker of platelet activation[55,56]. However, it is not specific for platelets. It can also be shed from activated endothelial cells.

Glycocalicin

Glycocalicin is a carbohydrate-rich amino-terminal proteolytic fragment of GpIb. It is cleaved during platelet activation or during destruction by calpains and circulates in the plasma[57]. Glycocalicin levels in plasma are dependent on platelet count and platelet turnover. Plasma glycocalicin was measured with an enzyme-immunoassay. The level is reported to be elevated in conditions associated with platelet destruction. Low glycocalicin levels are seen in patients with aplastic anemia or amegakaryocytic thrombocytopenia[58].

Flow cytometry-based platelet function tests

The flow cytometer is being increasingly used to detect activated platelets. It has several advantages[59,60]. It can be used to detect platelets because of the smaller size of platelets than red blood cells and white blood cells. It can be performed in very small volumes of blood. Furthermore, availability of a large number of fluorescent antibodies, ligands and probes to assess expression of neoantigens, ligand binding and the calcium fluxes has rendered flow cytometry to be very versatile for detecting different platelet markers. Another advantage is that platelets can be analysed in their native plasma milieu and do not require isolation. The flow cytometry can be used to evaluate platelet function in severe thrombocytopenic patients. Furthermore, platelets can be fixed before or after antibody labelling. Flow cytometry has also the capacity for detecting circulating activated platelets and leukocyte–platelet aggregates, as well as to enumerate platelet-derived microvesicles[60]. As in the measurement of soluble markers, utmost care is necessary to draw blood with a clean venipuncture and samples should be processed immediately to avoid activation in vitro.

Detection of circulating activated platelets

Platelet activation results in the appearance of new markers on the platelet surface. These markers include changes in the conformation of GPIIb–IIIa detectable by monoclonal antibodies (e.g. PAC1 epitope), changes in GPIIb–IIIa induced by ligands (e.g. LIBS1 and LIBS6) or GPIIb–IIIa-induced conformational changes in fibrinogen (e.g. 9F9, F26)[61–65]. In addition, activated platelet surface acquires additional membrane markers from the intra-platelet granules. These markers include P-selectin, thrombospondin, multimerin (from alpha granules), CD 63 and LAMP-1 (from lysosome)[66–70]. Furthermore, the exposure of anionic phospholipid due to transbilayer movement can also be used as a marker for platelet activation[71]. The anionic phospholipid can be detected as annexin V positive platelets. Of these markers, GPIIb–IIIa detected by PAC1 antibodies and CD62 (P-selectin) have been widely used in clinical studies to detect activated platelets in the circulation. These studies have shown the presence of circulating activated platelets in coronary artery disease[72] and following coronary angioplasty[73]. The presence of activated platelets in the circulation may predict subsequent occurrence of ischemic events[74].

Detection of platelet-derived microvesicles

Externalization of anionic phospholipid during platelet activation is accompanied by shedding of phosphatidylserine-rich microvesicles[75]. In the vesiculation process, small areas of the surface membrane are shed in a true budding process. By freeze fracture electron microscopy, these microparticles have been shown to possess bilayer structure. These vesicles are enriched in platelet procoagulant activity and contain platelet membrane GP Ib, IIb, IIIa and IV as well as membrane-bound factor Va[76,77]. These microvesicles possess procoagulant activity as intact platelets and may play a physiological role in providing a catalytic surface for optimal thrombin formation. Shear stress is also a potent stimulus for microvesiculation[78,79]. Platelet-derived microvesicles can be detected by flow cytometry by gating the GPIIb–IIIa positive particles and measuring the annexin V positivity[70]. Increased microvesicles have been detected in circulation in patients with

Cambridge Books Online © Cambridge University Press, 2009

disseminated intravascular coagulation[80], heparin-induced thrombocytopenia[81] and 'antiphospholipid antibody syndrome'[82], transient ischemic attacks[83], and thrombotic thrombocytopenic purpura[84]. These associations suggest that, while they may be necessary for normal hemostasis, elevated microvesicle concentrations could predispose to thrombosis.

Detection of circulating platelet aggregates

Circulating platelet aggregates can be measured by flow cytometry. The existence of circulating platelet aggregate (CPA) was first proposed by Wu and Hoak[85]. They developed a formalin fixing method for measuring CPA in patients with myocardial infarction and stroke[86]. This method was widely used in the 1970s by many laboratories worldwide. However, the method is semiquantitative. Recent measurements of CPA by flow cytometry have confirmed the presence of CPA in patients with arterial thrombosis[87]. Flow cytometry for CPA provides a quantitative measurement of the number of aggregates as well as the size of aggregates. On the flow cytometer, the aggregates appear as a smearing of the platelets in the upper right quadrants[88]. Activated platelets also interact with neutrophils and monocytes to form heterotypic aggregates. Using platelet-specific and leukocyte-specific monoclonal antibodies these aggregates can be detected in the peripheral blood. These aggregates are increased in myocardial infarction[89], unstable angina[90], coronary artery disease[91] and cardiopulmonary by pass[92]. It will be interesting to determine by prospective studies whether CPA, a reflection of SPA, is a risk factor of CHD and stroke.

Measurement of platelet procoagulant activity

As discussed earlier, during secondary hemostasis, activated platelets provide a highly efficient procoagulant surface[93]. Anionic phospholipids translocate from the inner leaflet of the platelet membrane to the outer leaflet and there support the binding and activation of vitamin K-dependent coagulation proteins: factors VII, IX, and X and prothrombin (see Ref. 5 for a general review of the coagulation pathways). In addition, factor V which is present in the α-granules of the platelets, is activated and the resulting factor Va, provides a binding site for factor Xa[94]. The complex of factor Xa–Va assembled on the anionic phospholipid surface of platelets is called the prothrombinase complex. Zwaal et al. have measured this procoagulant activity, i.e. the capacity to catalyze prothrombin or factor X activation in a quantitative manner in kinetic assays[95]. However, this assay is technically demanding and the product of the reaction, thrombin, is a potent platelet activator[96]. As platelets become activated during the assay, the thrombin generation rate increases, and measurement of the initial rate of thrombin generation is often unreliable. Jesty et al. have used acetylated prothrombin in a modified assay[97]. This modified prothrombin is activated by prothrombinase, but is generated as an abnormal thrombin that does not activate platelets but still retains amidolytic activity towards small synthetic substrates. This modification allows quantitative measurement of platelet procoagulant activity without the feedback effect of thrombin. The clinical utility of this assay has not been studied.

Test of platelet gene polymorphism

There is increasing evidence that platelet function is influenced by polymorphisms of platelet membrane glycoproteins notably GPIIb–IIIa, GPIb and GPIaIIa[98]. The data so far reported represent the tip of the iceberg. The rapid progresses in genomic polymorphism determinations provide an unheralded opportunity to investigate the influence of genome-wide polymorphisms of functionally important genes on platelet function and platelet function tests. Since DNA polymorphism techniques are not subjected to in vitro variations encountered with almost all in vitro platelet function tests, genetic testing should provide valuable information regarding interindividual variation in platelet function. The genetic testing will be potentially useful in assessing risk of arterial thrombosis and response to antiplatelet drugs.

A commonly used method for detecting single nucleotide polymorphism (SNP) is amplification of the region of polymorphism by polymerase chain reaction and digest the amplified DNA with an appropriate restriction enzyme. SNP may create a new restriction site or obliterate an existing restriction site in the amplified region. In both cases, restriction enzyme digestion will yield a single DNA vs. two bands on gel eletrophoresis. Under certain circumstances under which polymorphism does not create or obliterate a restriction site, the SNP is detected by single strand conformation polymorphism (SSCP). Platelet glycoproteins are highly polymorphic and several of the polymorphisms have been identified as platelet alloantigens[99]. One of the alloantigens, P1A1 and P1A2 (or HPA-1) is identified as a SNP of GPIIIa (Leu33Pro)[100]. This polymorphism was shown in a non-prospective study to be associated with myocardial infarction especially in younger individuals (<60 years old)[101]. However, prospective studies did not find an association of this polymorphism with coronary heart disease[102,103]. Polymorphisms in other alloantigens, i.e. HPA-2 (GP1b Met 145 Thr), HPA-3 (GPIIIa Ile 843 Ser) and HPA-5 (GPIa, Glu505Lys) were not found to be asso-

Cambridge Books Online © Cambridge University Press, 2009

ciated with an increased risk of stroke[104]. Two linked allelic SNP of GPIa (nucleotide 807 C→T, 873 G→A) were found to influence the expression of GPIa integrin. Individuals with C807G873 allele have lower levels of GPIaIIa[105]. The T807 allele was found to be associated with non-fatal myocardial infarction in younger patients[106]. However, it is unclear that this polymorphism predicts the risk of development of myocardial infarction in healthy subjects. Variable numbers tandem repeat (VNTR) polymorphism of GPIbα gene[107] has been shown to be associated with arterial thrombotic disease[108] but the predictive value remains unknown. Overall, single SNP or VNTR polymorphism of GPIIb–IIIa, GPIb–V–IX and GPIaIIa appears to have limited association, if any, with coronary heart disease or stroke. It has become increasingly recognized that the biological effect of a single polymorphism of a given gene is small. It may be necessary to study the association of multiple polymorphisms with arterial thrombosis. This can be done by determining all known polymorphisms of candidate platelet glycoprotein genes in cases and controls and analysing the allellic association with thrombotic events. Alternatively, all the polymorphism sites of a given gene are identified by oligonucleotide microarray techniques in well-defined cases and controls and the association of alleles with risk of coronary heart disease and stroke analysed. These approaches are now being developed and tested but their value in assessing risk of arterial thrombosis or drug response has not been reported.

Conclusion

Availability of new and more specific methods for measuring platelet aggregability and activated platelets provides an opportunity to investigate the relationship of altered platelet function with human thrombotic and bleeding disorders. Now there are methods available for providing answers to a question that has intrigued investigators for decades: do hyperreactive platelets predispose individuals to arterial and venous thrombosis? Tests such as flow cytometry for detecting activated platelets and circulating platelet aggregates, enzyme immunoassays for measuring plasma βTG, and urinary 11-dehydrothromboxane B_2 and genetic tests for platelet gene polymorphism will provide reliable information when implemented in well-designed case-control and population-based case-cohort studies. These tests will also provide useful information regarding the influence of genetic and environmental factors on risk of thrombosis. Another potential utility of these tests is to monitor the effect (and side effects) of antiplatelet agents.

References

1 Holvoet P, Collen D. Thrombosis and atherosclerosis. *Curr Opin Lipidol* 1997; 8: 320–328.

2 Ruggeri ZM. Old concepts and new developments in the study of platelet aggregation. *Clin Invest* 2001; 105: 699–701.

3 Andrews RK, Shen Y, Gardiner EE, Dong JF, Lopez JA, Berndt MC. The glycoprotein Ib-IX-V complex in platelet adhesion and signaling. *Thromb Haemost* 1999; 82: 357–364.

4 Shattil SJ. Signaling through platelet integrin alpha IIb beta 3: inside-out, outside-in, and sideways. *Thromb Haemost* 1999; 82: 318–325.

5 Brass LF, Hoxie JA, Manning D. Signaling through G proteins and G protein-coupled receptors during platelet activation. *Thromb Haemost* 1993; 70: 217–223.

6 Watson SP. Collagen receptor signaling in platelets and megakaryocytes. *Thromb Haemost* 1999; 82: 365–376.

7 Ruggeri ZM. Mechanisms initiating platelet thrombus formation. *Thromb Haemost* 1997; 78: 611–616.

8 Frojmovic MM. Platelet aggregation in flow: differential roles for adhesive receptors and ligands. *Am Heart J* 1998; 135: S119–131.

9 Kroll MH, Hellums JD, McIntire LV, Schafer AI, Moake JL. Platelets and shear stress. *Blood* 1996; 88: 1525–1541.

10 Bray PF. Platelet glycoprotein polymorphisms as risk factors for thrombosis. *Curr Opin Hematol* 2000; 7: 284–289.

11 Di Minno G, Piemontino U, Cerbone AM. Nutrition and thrombogenic factors. *Nutr Metab Cardiovasc Dis* 1999; 9: Suppl: 16–20.

12 Thiagarajan P, Wu KK. Mechanism of antithrombotic drugs. *Adv Pharmacol* 1999; 46: 297–324.

13 Mielke CH. Measurement of the bleeding time. *Thromb Haemost* 1984; 52: 210–211.

14 Harker LA, Slichter SJ. The bleeding time as a screening test for evaluation of platelet function. *N Engl J Med* 1972; 287: 155–159.

15 Rodgers RP, Levin J. A critical reappraisal of the bleeding time. *Semin Thromb Haemost* 1990; 16: 1–20 (Review).

16 Kratzer MA, Born GV. Simulation of primary haemostasis *in vitro*. *Haemostasis* 1985; 15: 357–362.

17 Israels SJ, Cheang T, McMillan-Ward EM, Cheang M. Evaluation of primary hemostasis in neonates with a new in vitro platelet function analyzer. *J Pediatr* 2001; 138: 116–119.

18 Lasne D, Fiemeyer A, Chatellier G, Chammas C, Baron JF, Aiach M. A study of platelet functions with a new analyzer using high shear stress (PFA 100) in patients undergoing coronary artery bypass graft. *Thromb Haemost* 2000; 84: 794–799.

19 Ortel TL, James AH, Thames EH, Moore KD, Greenberg CS. Assessment of primary hemostasis by PFA-100 analysis in a tertiary care center. *Thromb Haemost* 2000; 84: 93–97.

20 Homoncik M, Jilma B, Hergovich N, Stohlawetz P, Panzer S, Speiser W. Monitoring of aspirin (ASA) pharmacodynamics with the platelet function analyzer PFA-100. *Thromb Haemost* 2000; 83: 316–321.

Cambridge Books Online © Cambridge University Press, 2009

21 Gorog P, Ahmed A. Haemostatometer: a new *in vitro* technique for assessing haemostatic activity of blood. *Thromb Res* 1984; 34: 341–357.
22 Li CK, Hoffmann TJ, Hsieh PY, Malik S, Watson WC. The xylum clot signature analyzer: a dynamic flow system that simulates vascular injury. *Thromb Res* 1998; 92: S67–77.
23 Igawa T, Kornhauser R, Cilla DD, King JO, Kambayashi J. Evaluation of the clot signature analyzer as a hemostasis test in healthy volunteers exposed to low doses of aspirin. *Clin Appl Thromb Hemost* 1999; 5: 117–121.
24 Born GVR. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature* 1984; 194: 927–929.
25 Zwierzina WD, Kunz F. A method of testing platelet aggregation in native whole blood. *Thromb Res* 1985; 38: 91–100.
26 Skoza L, Zucker MB, Jerushalmy Z, Grant R. Kinetic studies of platelet aggregation induced by adenosine diphosphate and its inhibition by chelating agents, guanidino compounds, and adenosine. *Thromb Diath Haemorrh* 1967; 18: 713–725.
27 Zucker MB. Platelet aggregation measured by the photometric method. *Meth Enzymol* 1989; 169: 117–133.
28 Wu KK, Hoak JC. A new method for the quantitative detection of platelet aggregates in patients with arterial insufficiency. *Lancet* 1974; 2: 924–926.
29 Uchiyama S, Takeuchi M, Osawa M et al. Platelet function tests in thrombotic cerebrovascular disorders. *Stroke* 1983; 14: 511–517.
30 Trip MD, Cats VM, van Capelle FJ, Vreeken J. Platelet hyperreactivity and prognosis in survivors of myocardial infarction. *N Engl J Med* 1990; 322: 1549–1554.
31 Haeram JW. Platelet aggregates in intramyocardial vessels of patients dying suddenly and unexpectedly of coronary artery disease. *Atherosclerosis* 1972; 15: 199–213.
32 Robert A, Olmer M, Sampol J, Gugliotta JE, Casanova P. Clinical correlation between hypercoagulability and thrombo-embolic phenomena. *Kidney Int* 1987; 31: 830–835.
33 Wu KK. Platelet activation mechanisms and markers in arterial thrombosis. *J Intern Med* 1996; 239: 17–34.
34 Veyradie, A, Fressinaud E, Meyer D. Laboratory diagnosis of von Willebrand disease. *Int J Clin Lab Res* 28: 201.
35 Miller JL, Kupinski JM, Castella A, Ruggeri ZM. von Willebrand factor binds to platelets and induces aggregation in platelet-type but not type IIB von Willebrand disease. *J Clin Invest* 1983; 72: 1532–1542.
36 Ingerman-Wojenski C, Smith JB, Silver MJ. Evaluation of electrical aggregometry: comparison with optical aggregometry, secretion of ATP, and accumulation of radiolabeled platelets. *J Lab Clin Med* 1983; 101: 44–52.
37 Abbate R, Boddi M, Prisco D, Gensini GF. Ability of whole blood aggregometer to detect platelet hyperaggregability. *Am J Clin Pathol* 1990; 94: 525–526.
38 Smith JW, Steinhubl SR, Lincoff AM et al. Rapid platelet-function assay: an automated and quantitative cartridge-based method. *Circulation* 1999; 99: 620–625.
39 Tsuji S, Sugimoto M, Miyata S, Kuwahara M, Kinoshita S, Yoshioka A. Real-time analysis of mural thrombus formation in various platelet aggregation disorders: distinct shear-dependent roles of platelet receptors and adhesive proteins under flow. *Blood* 1993; 94: 968–975.
40 Goto S, Sakai H, Goto M et al. Enhanced shear-induced platelet aggregation in acute myocardial infarction. *Circulation* 1999; 99: 608–613.
41 Konstantopoulos K, Grotta JC, Sills C, Wu KK, Hellums JD. Shear-induced platelet aggregation in normal subjects and stroke patients. *Thromb Haemost* 1995; 74: 1329–1334.
42 Sakariassen KS, Muggli R, Baumgartner HR. Measurements of platelet interaction with components of the vessel wall in flowing blood. *Meth Enzymol* 1989; 169: 37–70.
43 Hubbell JA, McIntire LV. Visualization and analysis of mural thrombogenesis on collagen, polyurethane and nylon. *Biomaterials* 1986; 7: 354–363.
44 Kaplan KL. Proteins secreted by platelets: significance in detecting thrombosis. *Adv Exp Med Biol* 1978; 102: 105–119.
45 Kaplan KL, Owen J. Plasma levels of beta-thromboglobulin and platelet factor 4 as indices of platelet activation *in vivo*. *Blood* 1981; 57: 199–202.
46 Gershlick AH. Are there markers of the blood-vessel wall interaction and of thrombus formation that can be used clinically?. *Circulation* 1990; 81: 128–134.
47 Papp AC, Hatzakis H, Bracey A, Wu KK. ARIC hemostasis study—I. Development of a blood collection and processing system suitable for multicenter hemostatic studies. *Thromb Haemost* 1989; 61: 15–19.
48 Ho CH, Hu HH, Wong WJ. The serial hemostasis-related changes in patients with cerebral infarction: comparison between progressing and non-progressing stroke. *Thromb Res* 1989; 56: 635–647.
49 Ghaddar HB, Cortes J, Salomaa V et al. Correlation of specific platelet activation markers with carotid arterial wall thickness. *Thromb Haemost* 1995; 74: 943–948.
50 FitzGerald GA, Pedersen AK, Patrono C. Analysis of prostacyclin and thromboxane biosynthesis in cardiovascular disease. *Circulation* 1983; 67: 1174–1177.
51 Weber C, Holler M, Beetens J, De Clerck F, Tegtmeier F. Determination of 6-keto-PGF1 alpha, 2,3-dinor-6-keto-PGF1 alpha, thromboxane B2, 2,3-dinor-thromboxane B2, PGE2, PGD2 and PGF2 alpha in human urine by gas chromatography-negative ion chemical ionization mass spectrometry. *J Chromatogr* 1991; 562: 599–611.
52 Mene P, Pecci G, Cinotti GA, Pugliese G, Pricci F, Pugliese F. Eicosanoid synthesis in peripheral blood monocytes: a marker of disease activity in lupus nephritis. *Am J Kidney Dis* 1998; 32: 778–784.
53 Wagner DD. The Weibel–Palade body: the storage granule for von Willebrand factor and P-selectin. *Thromb Haemost* 1993; 70: 105–110.
54 Ishiwata N, Takio K, Katayama M et al. Alternatively spliced isoform of P-selectin is present *in vivo* as a soluble molecule. *J Biol Chem* 1994; 269: 23708–23715.
55 Ferroni P, Basili S, Martini F et al. Soluble P-selectin as a

Cambridge Books Online © Cambridge University Press, 2009

marker of platelet hyperactivity in patients with chronic obstructive pulmonary disease. *J Investig Med* 2000; 48: 21–27.
56 Semenov AV, Romano, YA, Loktionova SA et al. Production of soluble P-selectin by platelets and endothelial cells. *Biochemistry (Mosc)* 1999; 64: 1326–1335.
57 Coller BS, Kalomiris E, Steinberg M, Scudder LE. Evidence that glycocalicin circulates in normal plasma. *J Clin Invest* 1984; 73: 794–799.
58 Steinberg MH, Kelton JG, Coller BS. Plasma glycocalicin. An aid in the classification of thrombocytopenic disorders. *N Engl J Med* 1987; 317: 1037–1042.
59 Shattil SJ, Cunningham M, Hoxie JA. Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. *Blood* 1987; 70: 307–315.
60 Michelson AD, Barnard MR, Krueger LA, Frelinger AL 3rd, Furman MI. Evaluation of platelet function by flow cytometry. *Methods* 2000; 21: 259–270.
61 Ginsberg MH, Frelinger AL, Lam SC et al. Analysis of platelet aggregation disorders based on flow cytometric analysis of membrane glycoprotein IIb–IIIa with conformation-specific monoclonal antibodies. *Blood* 1990; 76: 2017–2023.
62 Abrams CS, Ellison N, Budzynski AZ, Shattil SJ. Direct detection of activated platelets and platelet-derived microparticles in humans. *Blood* 1990; 75: 128–138.
63 Frelinger AL 3rd, Cohen I, Plow EF et al. Selective inhibition of integrin function by antibodies specific for ligand-occupied receptor conformers. *J Biol Chem* 1990; 265: 6346–6352.
64 Gralnick HR, Williams SB, McKeown L et al. Endogenous platelet fibrinogen: its modulation after surface expression is related to size-selective access to and conformational changes in the bound fibrinogen. *Br J Haematol* 1992; 80: 347–357.
65 Zamarron C, Ginsberg MH, Plow EF. A receptor-induced binding site in fibrinogen elicited by its interaction with platelet membrane glycoprotein IIb–IIIa. *J Biol Chem* 1991; 266: 16193–16199.
66 Nieuwenhuis HK, van Oosterhout JJ, Rozemuller E, van Iwaarden F, Sixma JJ. Studies with a monoclonal antibody against activated platelets: evidence that a secreted 3,000-molecular weight lysosome-like granule protein is exposed on the surface of activated platelets in the circulation. *Blood* 1987; 70: 838–845.
67 Febbraio M, Silverstein R. Identification and characterization of LAMP-1 as an activation-dependent platelet surface glycoprotein. *J Biol Chem* 1990; 265: 18531–18537.
68 Boukerche H, McGregor JL. Characterization of an anti-thrombospondin monoclonal antibody (P8) that inhibits human blood platelet functions. Normal binding of P8 to thrombin-activated Glanzmann thrombasthenic platelets. *Eur J Biochem* 1988; 171: 383–392.
69 Hayward CP, Smith JW, Horsewood P, Warkentin TE, Kelton JG. p-155, a multimeric platelet protein that is expressed on activated platelets. *J Biol Chem* 1991; 266: 7114–7120.
70 Henn V, Slupsky JR, Grafe M et al. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 1998; 391: 591–594.
71 Thiagarajan P, Tait JF. Collagen-induced exposure of anionic phospholipid in platelets and platelet derived microparticles. *J Biol Chem* 1991; 266: 24302–23305.
72 Furman MI, Benoit SE, Barnard MR et al. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. *J Am Coll Cardiol* 1998; 31: 352–358.
73 Scharf RE, Tomer A, Marzec UM, Teirstein PS, Ruggeri ZM, Harker LA. Activation of platelets in blood perfusing angioplasty-damaged coronary arteries. Flow cytometric detection. *Arterioscler Thromb* 1992; 12: 1475–1487.
74 Tschoepe D, Schultheiss HP, Kolarov P et al. Platelet membrane activation markers are predictive for increased risk of acute ischemic events after PTCA. *Circulation* 1993; 88: 37–42.
75 Sandberg H, Andersson LO, Hoglund S. Isolation and characterization of lipid-protein particles containing platelet factor 3 released from human platelets. *Biochem J* 1982; 203: 303–311.
76 Bode AP, Sandberg H, Dombros, FA, Lentz BR. Association of factor V activity with membranous particles released from human platelets: requirement for platelet stimulation. *Thromb Res* 1985; 39: 49–61.
77 Sims PJ, Wiedmer T, Esmon CT, Weiss HJ, Shattil SJ. Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome: an isolated defect in platelet procoagulant activity. *J Biol Chem* 1989; 264: 17049–17057.
78 Miyazaki Y, Nomura S, Miyake T et al. High shear-stress can initiate platelet aggregation and shedding of procoagulant containing microparticles. *Blood* 1996; 88: 3456–3464.
79 Chou T, Hellum DA, Thiagrajan P. Synergistic interaction of thrombin and shear stress on microvesiculation of platelets. *J Lab Clin Med* 2000; 135: 66–72.
80 George GN, Picket, EB, Sauceman S et al. Platelet surface glycoproteins. Studies on resting and activated platelet and platelet membrane particles in normal subjects and observation in patients during adult respiratory distress and cardiac surgery. *J Clin Invest* 1986; 78: 340–346.
81 Warkentin TE, Hayword CPM, Santos AV, Kelton JG. Platelet-derived microvesicles are found *in vivo* with heparin-induced thrombocytopenia. *Thromb Haemost* 1993; 69: 912 (Abstract).
82 Galli M, Grassi A, Barbui A. Platelet-derived microvesicles in patients with antiphospholipid antibodies. *Thromb Haemost* 1993; 69: 912 (Abstract).
83 Lee YJ, Jy W, Horstman LL et al. Elevated platelet microparticles in transient ischemic attacks, lacunar infarcts and multi-infarct dementia. *Thromb Res* 1993; 72: 295–304.
84 Kelton JG, Warkentin TE, Hayword CPM, Murphy WG, Moore JC. Calpain activity in patients with thrombocytopenic purpura is associated with platelet microparticles. *Blood* 1992; 80: 2246–2250.
85 Wu KK, Hoak JC. A new method for the quantitative detection

Cambridge Books Online © Cambridge University Press, 2009

of platelet aggregates in patients with arterial insufficiency. *Lancet* 1974; ii: 924–926.

86 Wu KK, Hoak JC. Increased platelet aggregates in patients with transient ischemic attacks. *Stroke* 1975; 6: 521–524.

87 Konstantopoulos K, Grotta JC, Sills C, Wu KK, Hellums JD. Shear-induced platelet aggregation in normal subjects and stroke patients. *Thromb Haemost* 1995; 74: 1329–1334.

88 Chow TW, Turner NA, Chintagumpala M et al. Increased von Willebrand factor binding to platelets in single episode and recurrent types of thrombotic thrombocytopenic purpura. *Am J Hematol* 1998; 57: 293–302.

89 Gawaz M, Reininge, A, Neumann F. Platelet function and platelet-leukocyte adhesion in symptomatic coronary heart disease. Effects of intravenous magnesium. *Thromb Res* 1996; 83: 341–986.

90 Ott I, Neumann FJ, Gawaz M, Schmitt M, Schomig A. Increased neutrophil-platelet adhesion in patients with unstable angina. *Circulation* 1996; 94: 1239–1246.

91 Furman MI, Benoit SE, Barnard MR et al. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. *J Am Coll Cardiol* 1998; 31: 352–358.

92 Rinder CS, Bona, JL, Rinder HM, Mathew J, Hines R, Smith BR. Cardiopulmonary bypass induces leukocyte-platelet adhesion. *Blood* 1992; 79: 1201–1205.

93 Solum NO. Procoagulant expression in platelets and defects leading to clinical disorders. *Arterioscler Thromb Vasc Biol* 1999; 19: 2841–2846.

94 Bevers EM, Rosing J, Zwaal RF. Development of procoagulant binding sites on the platelet surface. *Adv Exp Med Biol* 1985; 192: 359–371.

95 Bevers EM, Comfurius P, van Rijn JL, Hemke, HC, Zwaal RF. Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. *Eur J Biochem* 1982; 122: 429–436.

96 Swords NA, Mann KG. The assembly of the prothrombinase complex on adherent platelets. *Arterioscler Thromb* 1993; 13: 1602–1612.

97 Jesty J, Bluestein D. Acetylated prothrombin as a substrate in the measurement of the procoagulant activity of platelets: elimination of the feedback activation of platelets by thrombin. *Anal Biochem* 1999; 272: 64–70.

98 Bray PF. Platelet glycoprotein polymorphisms as risk factors for thrombosis. *Curr Opin Hematol* 2000; 7: 284–289.

99 Newman PJ, Valentin N. Human platelet alloantigens: recent findings, new perspectives. *Thromb Haemost* 1995; 74: 234–239.

100 Goldberger A, Kolodziej M, Poncz M, Bennett JS, Newman PJ. Effect of single amino acid substitutions on the formation of the PlA and Bak alloantigenic epitopes. *Blood* 1991; 78: 681–687.

101 Weiss EJ, Bray PF, Tayback, M et al. A polymorphism of a platelet glycoprotein receptor as an inherited risk factor for coronary thrombosis. *N Engl J Med* 1996; 334: 1090–1094.

102 Ridker PM, Hennekens CH, Schmitz C, Stampfer MJ, Lindpaintner K. PIA1/A2 polymorphism of platelet glycoprotein IIIa and risks of myocardial infarction, stroke, and venous thrombosis. *Lancet* 1997; 349: 385–388.

103 Aleksic N, Juneja H, Folsom AR, Ahn C, Boerwinkle E, Chambless LE, Wu KK. Platelet Pl(A2) allele and incidence of coronary heart disease: results from the Atherosclerosis Risk In Communities (ARIC) study. *Circulation* 2000; 102: 1901–1905.

104 Sperr WR, Huber K et al. Inherited platelet glycoprotein polymorphisms and a risk for coronary heart disease in young central Europeans. *Thromb Res* 1998; 90: 117–123.

105 Kunicki M, Savage B, Nugent DJ, Santoso S, Ruggeri ZM, Kunicki TJ. Nucleotide polymorphisms in the alpha2 gene define multiple alleles that are associated with differences in platelet alpha2 beta1 density. *Blood* 1998; 92: 2382–2388.

106 Santoso S, Kunicki TJ, Kroll H, Haberbosch W, Gardemann A. Association of the platelet glycoprotein Ia C807T gene polymorphism with nonfatal myocardial infarction in younger patients. *Blood* 1999; 93: 2449–2453.

107 Lopez JA, Ludwig EH, McCarthy BJ. Polymorphism of human glycoprotein Ib alpha results from a variable number of tandem repeats of a 13-amino acid sequence in the mucin-like macroglycopeptide region. Structure/function implications. *J Biol Chem* 1992; 267: 10055–10061.

108 Gonzalez-Conejero R, Lozano ML, Rivera J et al. Polymorphisms of platelet membrane glycoprotein Ib associated with arterial thrombotic disease. *Blood* 1998; 92: 2771–2776.

Cambridge Books Online © Cambridge University Press, 2009

31

Monitoring antiplatelet therapy

Dermot Cox and Desmond Fitzgerald

Centre for Cardiovascular Science, Royal College of Surgeons, Dublin, Ireland

Introduction

The introduction of glycoprotein (GP) IIb/IIIa antagonists as new and powerful antiplatelet agents has focused attention on the need for methods to monitor antiplatelet therapy. Administration of GPIIb/IIIa antagonists is complicated as they have a very steep dose–response relationship. Moreover, their therapeutic index is low, with the necessity for strong inhibition to see benefit but bleeding complications arising if inhibition is too severe. The traditional method of monitoring by platelet aggregometry has serious limitations and is being replaced by new, faster, more convenient and more sensitive assays.

Platelet function assays

Platelet assays are divided into two groups: physiological assays, which measure the ability of an antiplatelet agent to inhibit a known function of platelets or a biochemical assay, which measures a change in a surrogate platelet marker.

Physiological assays

A major physiological response of platelets to an agonist is to bind fibrinogen and aggregate. Alternatively, platelets can adhere to a matrix without prior activation. Physiological assays monitor platelet aggregation or adhesion. These assays require fresh platelets, specialist equipment and trained staff. While these assays are potentially useful as they provide information on any alteration in the physiological function of platelets, they may not detect more subtle changes indicative of altered platelet function.

Biochemical assays

Surrogate markers of platelet function may also be useful. These include assays that measure changes in a receptor upon binding a drug, assays that measure enzyme function or assays that measure changes in surface proteins upon activation. These assays are sensitive and can detect changes in platelets in the absence of an alteration in platelet aggregation. However, changes in these platelet assays are not necessarily reflected in changes of platelet function.

Some of the biochemical assays utilize flow cytometry to estimate the quantity of antigen per platelet. One advantage of flow cytometry is that individual platelets can be examined. On the other hand, quantification of the antigen is difficult. However, antigen quantification can now be performed using special calibration kits.

Agonists

The selection of platelet agonist needs to be matched to the drug under study. Agonists generate a response through two signalling systems. One is phospholipase A_2-dependent and utilizes cyclooxygenase and thromboxane synthase to produce the agonist thromboxane A_2 (see Chapter 16). The other is phospholipase C-dependent and leads to the activation of protein kinase C (see Chapter 16). Agonists can act via one or other pathway, and some agonists act via phospholipase A_2 at low concentration and phospholipase C at high concentration. Hence the term weak agonist for those using phospholipase A_2 only and strong agonists for those using phospholipase C with or without phospholipase A_2 activity[1].

Thrombin

Thrombin is a strong platelet agonist (see Chapter 8) and can generate a response when no other agonist can. Thrombin studies in plasma require an anticoagulant as it causes coagulation or the use of washed or gel-filtered platelets. The thrombin response occurs through two different mechanisms. In gel-filtered platelets, aggregation occurs with concentrations of 0.01–0.05 U/ml thrombin and is inhibited by anti-GPIb antibodies[2]. At concentrations above 0.2 U/ml, anti-GPIb antibodies have no effect and the response is dependent on the protease activated receptors 1 and 4[3]. In platelet-rich plasma higher concentrations of thrombin are needed due to neutralization of thrombin by antithrombin III. Here 0.2–0.4 U/ml is the lowest concentration that will induce platelet aggregation.

TRAP

As an alternative to thrombin, a thrombin receptor activating peptide (TRAP) can be used. This is the peptide exposed by thrombin on the amino-terminal of the PAR-1 receptor. TRAP is synthesized as the hexapeptide SFLLRN and is used in the range 5–20 µM. This mimics the action of thrombin on the protease activated receptor-1 without the problems of coagulation[3].

ADP

ADP (see Chapter 9) is normally used at two concentrations. At 3–5 µM ADP-induced aggregation is strongly inhibited by aspirin while at 20 µM it is unaffected. However, platelets lose their responsiveness to ADP after a few hours[4]. Platelet sensitivity can be prolonged by storing PRP at room temperature in a tube with minimum air space.

Collagen

There are two collagen receptors on platelets (see Chapter 11), the integrin $\alpha_2\beta_1$ and GPVI. $\alpha_2\beta_1$ signals via phospholipase A_2 and is inhibited by aspirin, while GPVI signals through phospholipase C and is unaffected by aspirin[5]. There are a number of different collagens with different affinities for the two receptors, thus, the effect of aspirin on collagen-induced aggregation depends on the source of collagen and the concentration used. If it is to be used, collagen from different suppliers should be tested for sensitivity.

Arachidonic acid

The action of phospholipase A_2 is to cleave arachidonic acid from the cell membrane and make it available as a substrate for COX (see Chapters 15 and 25). Thus, arachidonic acid is a useful agonist for studying the integrity of the cyclooxygenase pathway. Arachidonic acid can be obtained in the acid form soluble in organic solvents or as sodium arachidonate, which is water soluble.

U46619

U46619 is a thromboxane receptor agonist[6]. It is useful for selectively activating the thromboxane receptor independently of thromboxane synthesis. It can be used to detect inhibition by thromboxane receptor antagonists[7] and is not affected by aspirin.

Adrenaline

There are large variations in response to adrenaline and some people do not respond at all or need very high doses[8,9]. There can also be a lag in the response to adrenaline of up to several minutes. Failure to obtain a response to adrenaline could be due to the presence of an antiplatelet agent or just insensitivity to adrenaline.

Ristocetin

Ristocetin is an antibiotic that binds to vonWillebrand factor (vWf) allowing it to bind to GPIb (see Chapter 12). When paraformaldehyde-fixed platelets are used the response is agglutination rather than aggregation[10,11], is independent of GPIIb/IIIa and can be used as a measure of GPIb function.

Botrocetin

Botrocetin is a snake venom and another vWf modulator that can be used in agglutination studies[11,12] but its expense means that it is usually only used as a research tool.

Antiplatelet agents

Antiplatelet agents have different modes of action and knowledge of these is necessary to monitor therapy.

Cambridge Books Online © Cambridge University Press, 2009

Inhibitors of platelet agonist receptors

While platelets can be activated by many agonists, ADP receptors appear to be especially important probably due to the role of secreted ADP in amplifying the aggregation response (see Chapter 9). ADP receptor antagonists such as ticlopidine and clopidogrel[13] inhibit ADP-induced aggregation but not thrombin-induced aggregation (see Chapter 64). Thromboxane receptor antagonists on the other hand can inhibit arachidonic acid-induced aggregation but not aggregation due to high concentrations of ADP or thrombin[14].

Platelet signalling inhibitors

Platelet agonist receptors use two principle signalling pathways. Pharmacologically the most relevant is the phospholipase A_2 pathway, which generates thromboxane A_2 via cyclooxygenase from arachidonic acid (see Chapter 15). This pathway is inhibited by aspirin (see Chapter 61). Agonists such as collagen and thrombin use the phospholipase C pathway to signal and are not affected by aspirin[1]. Samples from patients on aspirin will not aggregate to arachidonic acid but will aggregate to high concentrations of thrombin, collagen and ADP.

GPIIb/IIIa receptor antagonists

Fibrinogen binding to its receptor, GPIIb/IIIa, is an essential step in platelet aggregation (see Chapter 13 and Chapter 63). Inhibitors of this receptor inhibit aggregation induced by all agonists[15]. However, they exhibit greater inhibition of a weak agonist like ADP compared with a strong agonist such as TRAP[16].

GPIb receptor antagonists

The interaction of GPIb with immobilized vWf under shear is one of the primary initiating steps in thrombus formation (see Chapter 12). There is on-going research to produce antagonists for this receptor but to date none have been developed clinically.

Agents which elevate cAMP

cAMP acts to inhibit platelet aggregation by all agonists[17]. This can be achieved by the use of prostacyclin analogues or phosphodiesterase inhibitors (see Chapter 20). Another antiplatelet agent that is used is dipyridamole. Its proposed mechanism of action is inhibition of adenosine uptake as well as phosphodiesterase activity[18]. Increased plasma levels of adenosine also results in increased levels of intracellular cAMP[19].

Platelet aggregation

The gold standard for monitoring antiplatelet therapy is aggregometry which is based on a turbidometric technique devised by Born in 1962[20]. Platelet aggregometry utilises the principle that the absorbance of a suspension is dependent on the number of particles rather than on their size. Thus, as aggregation occurs the number of particles decrease and absorbance decreases and is usually expressed as light transmission. Rather than use whole blood, platelet-rich plasma (PRP) is prepared by centrifugation of blood at a low speed (850 x *g* for 3 min or 100 x *g* for 10 min). This PRP is added to a cuvette with a stirrer and stirred (900–1200rpm) while agonist is added.

An issue with aggregation is what to measure. There is the option of measuring percent aggregation or the slope of the aggregation curve. There is also the question of whether to measure the extent of aggregation at a fixed time-point (e.g. 2 min) or maximum aggregation. These can be different as some agonists induce reversible aggregation (see Chapter 23). The advantage of using the slope rather than extent of aggregation is that it is independent of the time of measurement and of reversible aggregation.

Some agonists produce aggregation only after a lag time. This is noticeable with adrenaline and collagen. In the case of bacteria-induced aggregation the lag time can be more pronounced, 5 min with some strains of *Streptococcus sanguis*[21] and 15 min with others[22].

Thrombocytopenia

Aggregation may be influenced by the platelet count. Aggregometry requires platelet counts between 200 000 and 400 000 platelets/µl. Some researchers adjust the platelet count to ensure it is within these levels for the sake of uniformity[23]. There is evidence that platelet count may affect the degree of inhibition by GPIIb/IIIa antagonists[23]. Diluting a sample with a high platelet concentration is relatively simple although it requires a particle counter, which can be difficult to calibrate and maintain. It also assumes that all particles the size of platelets are in fact platelets which is not necessarily the case as particles such as cell fragments may have a similar size. Concentrating a dilute platelet sample is difficult without activating the sample.

Drug-induced thrombocytopenia can occur in response to antiplatelet agents (see section 3 chpt 6) and is a known complication with GPIIb/IIIa antagonists[24]. Patients on

Cambridge Books Online © Cambridge University Press, 2009

GPIIb/IIIa antagonists are often on heparin and either of these agents can cause thrombocytopenia (see section 3 chpt 6). If the platelet count is low it can be difficult to perform aggregation, however, platelet aggregometers will still give a response with counts as low as 50 000 platelets/μl although this response will be attenuated. An alternative is to use the single platelet count method or flow cytometry, which are not affected by platelet count.

Anticoagulant

There are many anticoagulants available today but the standard anticoagulant for platelet aggregometry is 3.8% sodium citrate. There are mixed reports on using antithrombins such as heparin, low molecular weight heparin, hirudin and PPACK as anticoagulants for aggregometry. Some authors suggest that they are suitable for aggregometry[25] while others have shown that some agonists are affected by their use[26]. Although blood for aggregation is often collected in sodium citrate, some GPIIb/IIIa antagonists show different potencies in citrate and heparin anticoagulated blood[27,28]. This appears to be due to chelation of calcium from GPIIb/IIIa and the resulting conformational change increases the affinity for eptifibatide. If physiological calcium levels are required for aggregation requiring the use of an antithrombin as anticoagulant it is important to choose the appropriate agonist, ideally high dose ADP, collagen or TRAP. The alternative is to re-calcify citrated PRP immediately prior to aggregation. This works well as aggregation occurs more rapidly than coagulation[26].

Reliability

There is a large variation in aggregation responses. In one study the standard deviation ranged from 3.6% to 7.7%, day-to-day variation accounted for 42% to 63% and variation between operators accounted for 1% to 33% of the total variation. Repeat measurements of a given sample by a given operator on a single day contributed 22% to 36% of the total variation[29]. These variations can be due to sample preparation, dietary factors including high levels of lipids in the sample, smoking, drinking and ageing of the sample.

Selectivity

A difficulty with platelet aggregation is the problem of identifying the drugs involved. Patients on GPIIb/IIIa antagonists are usually on aspirin. Some may have two different GPIIb/IIIa antagonists in their plasma: abciximab for use during intervention and non-peptide antagonist such as tirofiban for maintenance. Aggregation cannot distinguish between the different agents, only the total effect. However, use of a strong agonist like TRAP can select for actions due to a GPIIb/IIIa antagonist rather than those due to aspirin.

Platelet count

A platelet aggregometer measures the changes in light transmission when a suspension of platelets aggregate. However, its sensitivity is such that only aggregates of around eight platelets are detected. Using a particle counter to determine the platelet count before and after activation, it is possible to measure the loss of single platelets with a very high sensitivity. The inhibition of microaggregate formation has been used to study the effects of GPIIb/IIIa antagonists[28,30,31] and beraprost (prostacyclin analogue) was found to be between two and ten times more potent than in platelet aggregometry[32]. This assay can be used on thrombocytopenic samples unlike any of the functional assays such as light transmittance aggregometry. One advantage of the assay is that it requires no specialist equipment other than a particle counter, which is available in most hematology departments. The principle has also been used for the development of the ICHOR point-of-care assay[33].

Platelet function analyser (PFA-100™)

Recently a number of simpler systems have been devised to replace platelet aggregometry. The first to be introduced is the Platelet Function Analyzer PFA-100™, from Dade International, Miami, Florida, USA. This consists of a membrane that is coated with collagen–adrenaline or collagen–ADP. Whole blood is aspirated through a hole pierced in the membrane. The time to closure, which is due to the formation of a platelet plug, is a measure of platelet function[34].

This device has the advantage of using small volumes of whole blood directly applied to a cartridge. However, only cartridges with the supplied agonists can be used. Clinically it has been used to monitor GPIIb/IIIa antagonists[35] and aspirin[36,37].

Ultegra™ rapid platelet function analyser

This assay is based on TRAP-induced platelet aggregation. It consists of a cartridge containing blue beads coated with fibrinogen, a magnetic stirrer and TRAP. Whole blood is added to the cartridge and the resulting activated platelets bind to the coated beads forming aggregates. Aggregation

Cambridge Books Online © Cambridge University Press, 2009

is detected by monitoring the loss of the blue beads by the turbidometric method using filters to analyze the blue portion of the spectrum[38]. This device is made by Accumetrics Corporation, San Diego, CA, USA, and received FDA approval in 1999.

The advantage of this assay is that it only uses a small volume of whole blood and requires no sample preparation. It is also a bedside device, which makes testing convenient. However, it is only used for measuring inhibition by GPIIb/IIIa antagonists and only uses TRAP as the agonist. It has been successfully used to monitor abciximab[39,40] and orbofiban[41] levels in blood. The Ultegra™ gives results similar to platelet aggregation but the IC_{50} value tends to be lower.

Whole blood aggregometry

As its name implies this technique monitors platelet aggregation in whole blood. The extent of aggregation is determined by measuring changes in impedance across two wires as platelet aggregates form on them. These devices are manufactured by Chrono-Log Corporation, Havertown, PA, USA. This technique has the advantage of being rapid as there is no sample preparation required and is potentially more relevant physiologically as all the components of the blood are present. It has been reported to be more sensitive than aggregometry and it has been used successfully to monitor the effect of GPIIb/IIIa antagonists[42], aspirin[43], dipyridamole[44] and pentoxyfylline[45].

A modification of this technique is whole blood lumi-aggregometry. This allows for monitoring aggregation by the impedance method and ATP-secretion simultaneously[46]. Secretion is measured by a chemiluminescent assay using luciferase.

Flow cytometry

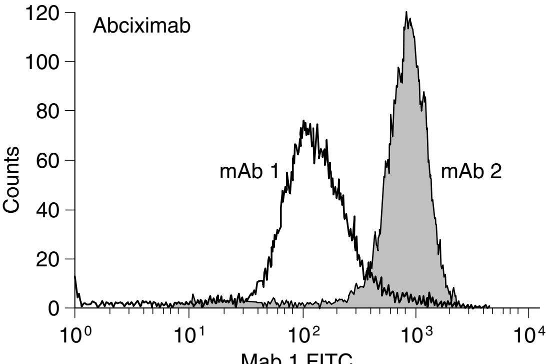
GPIIb/IIIa is capable of undergoing a number of conformational changes. Upon activation of the platelet by an agonist, GPIIb/IIIa changes from a resting to an active conformation. This new conformation can bind soluble fibrinogen. Fibrinogen binding to platelets can also be used to measure inhibition of GPIIb/IIIa. If PRP is activated and fixed the bound fibrinogen can be measured by flow cytometry. This has been used to monitor the effects of the GPIIb/IIIa antagonist YM337[47] and abciximab and orbofiban inhibition were measured using fluorescently labelled fibrinogen[48]. Another option is to utilise the conformational changes that fibrinogen undergoes when it binds to GPIIb/IIIa. These conformational changes expose neo-epitopes known as receptor-induced binding sites (RIBS)[49,50]. The antibody 9F9 recognises this RIBS epitope on fibrinogen and can be used to measure fibrinogen binding in whole blood[51].

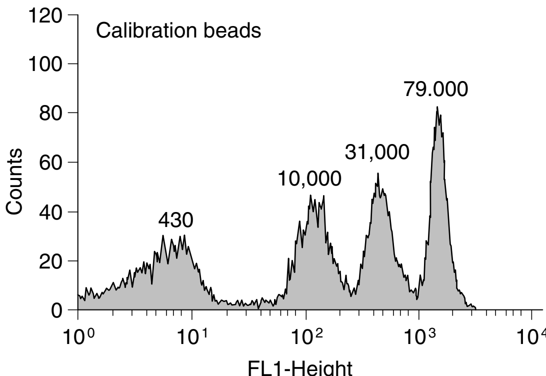
Activated GPIIb/IIIa undergoes a conformational change which can be recognized by the antibody PAC-1[52]. Fluorescently labelled PAC-1 has been used in flow cytometry to monitor inhibition of platelet activation by aspirin and clopidogrel[53] and inhibition of GPIIb/IIIa by the antagonists abciximab and orbofiban[48]. As well as conformational changes induced in the receptor by activation, the binding of antagonists to the receptor also induce conformational changes known as ligand-regulated binding sites (LRBS). One such LRBS occurs when ligands bind to GPIIb/IIIa and induce a conformational change in the receptor that results in the expression of neo-epitopes. These neo-epitopes are referred to as ligand-induced binding sites (LIBS)[54] and can be measured with anti-LIBS antibodies such as D3[55] and AP6[56] by flow cytometry. LIBS formation is not an all or none response and the degree of neo-epitope formation varies with different antagonists. However, LIBS formation, when it occurs at a reasonable level, can be used to estimate the number of occupied receptors on a platelet[57] and, used in combination with an antibody that recognizes all GPIIb/IIIa receptors, data on the free receptors and percent receptor occupancy can be obtained[58,59].

Another class of LRBS are epitopes whose expression is lost on ligand binding, i.e. ligand attenuated binding site (LABS). An antibody to a LABS has been used to develop the GPIIb/IIIa Receptor Occupancy assay from BioCytex, Marseille, France. This assay uses three antibodies: an isotype control and two anti-GPIIb/IIIa antibodies. One of these antibodies mAb2 recognises a drug-dependent LABS. A number of small molecule GPIIb/IIIa antagonists inhibit the binding of mAb2, although it is not inhibited by abciximab. The third antibody in the kit (mAb1) competes with abciximab and is not inhibited by small molecule antagonists. Together, these three antibodies can determine percent receptor occupancy[60].

One problem with flow cytometry is the inability to measure absolute binding of antibody rather than relative fluorescence. This problem has been overcome with the Receptor Occupancy Assay through its use of calibration beads. These are 2 μm beads with a known number of antibody molecules bound (there are four populations of beads). A labelled second antibody is used to stain the samples and beads. As a result it is possible to relate fluorescence to antibody molecules bound. This is known as quantitative fluorescence cytometry. As the antibodies are

Cambridge Books Online © Cambridge University Press, 2009





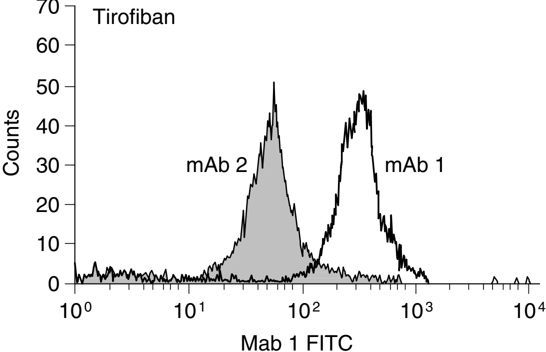


Fig. 31.1. Receptor occupancy assay. Binding of mAb 1 and mAb 2 to platelets from patients treated with abciximab or tirofiban. Also shown are the calibration beads showing four different populations of beads with different numbers of molecules bound.

differentially displaced by GPIIb/IIIa antagonists, mAb1 and mAb2 binding, after subtraction of the isotype binding, gives the number of free receptors and the number of occupied receptors. The ratio of the inhibited antibody to the uninhibited antibody (depending on the drug used) is the percentage inhibition (see Fig. 31.1).

The advantages of this assay are that it requires small amounts of whole blood (50μl) and does not require a baseline sample to estimate percentage inhibition. In addition it gives the number of free receptors as well, which may be more relevant than percent inhibition. If blood is activated with an agonist such as TRAP prior to use in the assay it allows comparison of inhibition in resting and activated samples. The receptor occupancy assay has been used to measure GPIIb/IIIa receptor occupancy by xemilofiban[61], orbofiban[16] and abciximab[62].

Thromboxane B_2

Measurement of thromboxane B_2 is especially useful for monitoring inhibition by aspirin. Thromboxane B_2 production is a measure of cyclooxygenase activity and can easily be measured by RIA/ELISA[63]. Measuring serum thromboxane B_2 by ELISA has been used to study the effects of aspirin[64], coated aspirin[65] and aspirin and nimesilide[66].

As an alternative to measuring serum thromboxane levels, the urinary metabolites of thromboxane can also be measured, in particular 2,3-dinor-TXB_2 and 11-dehydro-TXB_2 can be measured by gas chromatograph/tandem mass spectrometry (GC/MS) or ELISA[63,67]. The advantage of this is that urine is used rather than blood, and that platelet activation is not required (with serum thromboxane assays there is maximum activation of platelets). The assay is also suitable for large studies but does require specialist equipment. GC/MS analysis of urinary TXB_2 metabolites has been used to study the effects of aspirin[68], GPIIb/IIIa antagonists[57], L-arginine[69], nimesulide[66] and selective cyclooxygenase 2 inhibitors[70]. ELISA has been used to detect the actions of aspirin on urinary thromboxane B_2 levels[71].

There have been reports on resistance to the effects of aspirin[72]; however, results from platelet aggregometry can be misleading. True aspirin resistance only occurs when the drug does not completely inhibit thromboxane formation. Failure to inhibit platelet aggregation can be due to the involvement of the phospholipase C pathway in response to the agonist rather than a failure of aspirin to inhibit cyclooxygenase. Thus, measurement of COX activity by measuring thromboxane B_2 production is the most reliable method for detecting aspirin resistance.

cAMP and cGMP levels

Prostacyclin is a very potent inhibitor of platelet function and acts by elevating cAMP levels. Drugs which elevate cAMP such as the prostacyclin analogue beraprost and the phosphodiesterase inhibitor cilostazol can be monitored

Cambridge Books Online © Cambridge University Press, 2009

by measuring cAMP levels in platelets using an ELISA[73]. Equally, drugs which elevate cGMP (NO-donors) also inhibit platelet function and their actions can be monitored by ELISA/RIA for cGMP[74–76].

Platelet adhesion

Aside from platelet aggregation, platelets also adhere to the extracellular matrix. Adhesion to a fibrinogen-coated surface occurs in an activation independent manner and involves GPIIb/IIIa. Inhibition of adhesion can be used to monitor the levels of GPIIb/IIIa antagonist. A number of adhesion systems have been described in the literature. They can be static[77] or under flow conditions[78]. Under static conditions adhesion is predominantly a GPIIb/IIIa mediated event[77] and gives an indication of the levels of drug present. However, the IC_{50} values tend to be much higher than those obtained from platelet aggregometry. In some systems the shear stress can be regulated and increased to represent that found in arteries. This system depends largely on GPIb/IX/V rather than on GPIIb/IIIa[79]. The choice of substrate is also very important. However, there are no commercially available systems and those systems that are used require major investments in time and resources and are primarily research tools.

One system uses a chamber of known rheological properties containing a coverslip coated with ligand. Whole blood is pumped through this at different rates producing a range of different shear levels from those equivalent to venous conditions to those found in stenosed arteries. The coverslip is then removed, fixed, stained and the number of adherent platelets determined by microscopic examination[80]. Another shear system is the cone-and-plate viscometer[81]. The plate can be stained to allow the number of adherent particles and their sizes to be measured. Flow chambers can also be used in conjunction with real-time epifluorescence videomicroscopy[82].

Platelet adhesion has been applied in a number of clinical studies including studies on aprosulate, a novel synthetic glycosaminoglycan[83], the GPIIb/IIIa antagonist abciximab in combination with ticlopidine under shear[84], the GPIIb/IIIa antagonist YM337 under static conditions[47] and the GPIIb/IIIa antagonist eptifibatide under static and shear conditions[85].

Template bleeding time

The template bleeding time is the only in vivo assay of platelet function. Bleeding time is measured using a device that provides a standardized cut to the skin. The two principle methods are the Ivy and Simplate methods[86] and the bleeding time is the time taken for bleeding to stop (normally 8–12 min). It has been used to study the effects of the GPIIb/IIIa antagonist YM337[47]. At least 80% inhibition of platelet aggregation is necessary to cause a prolongation of bleeding time[87]. However, the effects of GPIIb/IIIa on bleeding time are not simply dependent on receptor occupancy but also on the nature of the antagonist. Some drugs have stronger effects on bleeding time than others relative to the levels of inhibition of platelet aggregation[88]. Other antiplatelet agents such as aspirin[36], clopidogrel[89], ticlopidine[90] and heparin[91] can also prolong the bleeding time.

The template bleeding time is not a sensitive assay, as high levels of inhibition are required to see an effect. However, as the principle adverse effect of anti-platelet agents is bleeding events, it is a good indicator of excessively high levels of inhibition. However, it is not popular with patients, as it is painful and can lead to scarring. The template bleeding time is also a poor predictor of bleeding events. In a clinical study with abciximab it was found that prolongation of the bleeding time was a poor predictor of bleeding events[92] and bleeding time was also shown to be a poor predictor of blood loss during surgery[93]. Thus, prolongation of bleeding time can indicate strong inhibition of platelet function but does not predict bleeding events.

Drug plasma level assays

Another approach is to directly measure plasma drug levels using ELISA, HPLC or mass spectrometry. However, with GPIIb/IIIa antagonists that have a fast on-rate and a slow off-rate such as abciximab[94] most of the drug is platelet bound and very little is free in the plasma. A similar situation arises with aspirin, which is an irreversible inhibitor of platelet cyclooxygenase[95], where the effects of the drug remain long after the drug is cleared.

Platelets and coagulation

Activated platelets undergo the membrane flip–flop, which exposes phospholipids, primarily phosphatidylserine, on their surface which are essential for the coagulation cascade. As a result the prothrombinase (FXa: Fva) and tenase (FIXa: FXa) complexes form on the platelet surface[96]. GPIIb/IIIa antagonists inhibit this procoagulant activity[97] which has led to the development of the Hemostatus test, which selectively measures the activity of GPIIb/IIIa antagonists[98].

Cambridge Books Online © Cambridge University Press, 2009

Another coagulation-based assay is the Clot Signature Analyzer™ from Xylum Corporation, New York. This assay uses non-anticoagulated whole blood and measures the time taken for platelets to occlude channels which are either uncoated (platelet hemostasis time, PHT) or coated with collagen (collagen-induced thrombus formation, CITF)[99]. Clinical studies report that the PHT is not affected by aspirin unlike the conventional bleeding time. The CITF however is more sensitive than collagen-induced aggregation but is considered overall to be unreliable[100].

The Sonoclot analyser™ from Sienco Inc., Morrison, Colorado, which measures plasma viscosity shows reasonable correlation with collagen-induced whole blood aggregation[101] although it was found to have a large coefficient of variance[102].

Clot retraction is mediated by a GPIIb/IIIa–fibrin interaction and involves platelet pseudopod contraction and is inhibited by GPIIb/IIIa antagonists[103]. Thromboelastography measures clot strength and is primarily dependent on fibrinogen and platelets. Tissue factor thromboelastography has been used to monitor levels of GPIIb/IIIa antagonists[104]. The Hemodyne™ (Hemodyne, Inc, Midlothian, VA) analyser which measures platelet-induced clot retraction[105] and a modified thrombelastograph (Haemoscope™, Skokie, IL), which measures clot strength, were found to be capable of monitoring activity of the GPIIb/IIIa antagonist abciximab[106].

Platelet function and environmental and genetic factors

The response of platelets to agonist stimulation is highly influenced by environmental factors. These can either inhibit the response to agonist, thereby mimicking the actions of a drug or else they can sensitize the platelets and as a result mask the effects of a drug.

Smoking

Smoking is one of the most common environmental factors influencing platelet function. Many researchers specifically exclude smokers when collecting blood from volunteers for studies. Smoking has been shown to increase the aggregability of platelets[107] and to increase spontaneous platelet aggregation (aggregation without the addition of agonist)[108]. Smokers were found to have increased levels of P-selectin on the platelet surface indicating platelet activation and this was not inhibited by low dose aspirin[109]. However, people who consider themselves to be non-smokers may be exposed to high levels of environmental tobacco smoke. Exposure to environmental tobacco smoke has been shown to increase the risk of cardiovascular disease[110,111]. Smoking increases thromboxane production as a consequence of platelet activation[112] and decreases prostacyclin production[113]. Aspirin was found to have a greater inhibitory effect on platelets from smokers than non-smokers, possibly reflecting altered thromboxane metabolism as in vitro thromboxane synthesis was decreased but urinary thromboxane secretion was increased[114].

Diet

Folic acid deficiency leads to enhanced platelet aggregation and thromboxane production[115]. Vitamin B12 supplementation can decrease platelet responses to platelet agonists. The flip side of this is that low intake of vitamin B12 especially in conjunction with hyperhomocysteinemia, which can occur in vegetarians, may be a risk factor for thrombosis[116]. Oxidant stress has also been shown to increase platelet reactivity suggesting that antioxidants may reduce platelet responses[117]. Vitamins C[118] and E[119] have been shown to inhibit platelet activation probably by acting as antioxidants, while vitamin B6 down regulates GPIIb levels on platelets[120]. Iron deficiency results in suppression of platelet aggregation[121,122] as does alcohol[123,124], garlic[125] and fish consumption[126]. Caffeine intake results in an increase in adenosine receptors with a resulting increase in cAMP levels[127].

Exercise

Exercise is known to increase platelet reactivity. This is probably due to increased catecholamine levels and is not inhibited by aspirin[128,129]. LDL lipid peroxides have also been implicated[130]. However, this increase in aggregability seems to be balanced by an increase in prostacyclin production in patients with a functional endothelium[131]. Exercise also increases sensitivity to antiplatelet agents[132].

Hormonal effects

GPIIb/IIIa on platelets from premenopausal women are more activatable than those of platelets from young men. An effect of menstrual cycle was also noted and changes in serum levels of estrogens and progestins were proposed as the mechanism of this effect[133]. Oral contraceptives are also known to increase the risk of thrombotic events, especially in women who smoke[113] or who are deficient in folic acid[115], although this may be a consequence of enhanced coagulation. However, physiological concentrations of estrogen were found to inhibit platelet aggregation and further inhibition was not seen in aspirin-treated platelets[134,135].

Cambridge Books Online © Cambridge University Press, 2009

Depression

There is a well-established link between depression and increase in platelet function[136] that is corrected by treatment with antidepressants[137].

Genetic

There is mixed evidence to suggest that polymorphisms of platelet receptors alter the response of platelets to agonists[138]. Some studies have shown an increased sensitivity of platelets with the PLA_2 polymorphism in GPIIIa to agonist stimulation compared with the PLA_1 variant[139,140] while others have shown no effect[141]. Platelets with the PLA_2 polymorphism have been shown to be less responsive to inhibition by the GPIIb/IIIa antagonists orbofiban[142] and to inhibition by aspirin[143], although they are more responsive to inhibition by estrogen[135]. A polymorphism in the collagen receptor has been shown to increase responsiveness to collagen by increasing the density of $\alpha_2\beta_1$ collagen receptors on the platelet surface and may be related to resistance to aspirin[144]. The Kozak polymorphism in GPIbα is associated with the levels of GPIb on the surface of platelets and may be implicated in enhanced platelet function[145] although it has been shown to have no effect on the incidence of myocardial infarction[146].

Conclusion

The importance of monitoring anti-thrombotic therapy has been seen with the oral GPIIb/IIIa antagonist trials. Failure to closely monitor drug levels can result in underdosing and therefore lack of efficacy, while overdosing means increased bleeding events for the patient. At present there are many assays available for monitoring antithrombotic therapy. The gold standard is still platelet aggregometry. It is flexible in that all anti-platelet agents can be measured and a range of agonists can be used. However, due to the specialist nature of the equipment, aggregometers are not widely available and it is time consuming due to sample preparation. There is also a lot of variation in the results. Newer rapid assays, some of which are bedside devices, have the advantage of using whole blood. This is more physiological and easier to perform as sample preparation is not necessary. The Ultegra™ is an effective system but at present only useful for monitoring anti-GPIIb/IIIa therapy. The PFA-100™ is also very effective and has a broader range of uses compared to the Ultegra™, although there are only two agonist combinations to use. Whole blood aggregometry offers the benefit of using whole blood but the flexibility of conventional aggregometry. One difficulty with the physiological assays is the choice of agonist and anticoagulant. Knowledge of the mechanism of action of the drug to be monitored is essential in selecting the appropriate agonist. For GPIIb/IIIa antagonists the issue of anticoagulant is also important. The quantitative fluorescence cytometry assay is the only assay not to require an agonist. While many of the assays described have been used to monitor the effects of antiplatelet agents some are difficult or unreliable or are only suitable as research tools.

Below are the three most widely used classes of antiplatelet agent and the most appropriate assays for monitoring them:

GPIIb/IIIa antagonists

Most assays are effective for monitoring GPIIb/IIIa antagonists. Whole blood assays such as the Ultegra™ and PFA-100™ are particularly suitable. If platelet aggregation is used the appropriate agonist is TRAP to selectively detect GPIIb/IIIa antagonist blockade. Drugs should be checked for the effects of calcium on their activity. If necessary, PPACK can be used as anticoagulant with TRAP as agonist. Quantitative fluorescence cytometry is also very effective for GPIIb/IIIa antagonists as it can give an indication of the number of free receptors available. If samples are stimulated with TRAP first it can give the number of free receptors on a maximally activated platelet. Apart from platelet aggregation all these methods use whole blood which makes them very convenient. As many hospitals have at least one flow cytometer the quantitative flow cytometry method is the only method which does not require specialist equipment. Another useful method is the single platelet count method. Not only does this use whole blood, it is the only method that is reliable when used on thrombocytopenic samples. Also cell counters are available in most hospitals and it is more sensitive than the other assays.

Aspirin

Aspirin is the most widely used antiplatelet agent and is often used in conjunction with other anti-platelet agents. The most sensitive and specific method to monitor inhibition by aspirin is to measure serum thromboxane levels by ELISA. This method can be used in the presence of other antiplatelet agents. In the absence of other antiplatelet agents, platelet aggregation can also be effective, especially if arachidonic acid is used as the agonist.

Ticlopidine/clopidogrel

These drugs are best monitored using platelet aggregation with ADP as the agonist. If aspirin is used as well, high dose ADP should be used to select for inhibition by these drugs.

Cambridge Books Online © Cambridge University Press, 2009

References

1 Siess W. Molecular mechanisms of platelet activation. *Physiol Rev* 1989; 69: 58–178.
2 Mazzucato M, Marco L, Masotti A, Pradella P, Bahou W, Ruggeri Z. Characterization of the initial alpha-thrombin interaction with glycoprotein Ib alpha in relation to platelet activation. *J Biol Chem* 1998; 273: 1880–1887.
3 Furman M, Liu L, Benoit S, Becker R, Barnard M, Michelson A. The cleaved peptide of the thrombin receptor is a strong platelet agonist. *Proc Nat Acad Sci USA* 1998; 95: 3082–3087.
4 Baurand A, Eckly A, Bari N et al. Desensitization of the platelet aggregation response to ADP: differential down-regulation of the P2Y1 and P2cyc receptors. *Thromb Haemost* 2000; 84: 484–491.
5 Inoue K, Ozaki Y, Satoh K et al. Signal transduction pathways mediated by glycoprotein Ia/IIa in human platelets: comparison with those of glycoprotein VI. *Biochem Biophys Res Commun* 1999; 256: 114–120.
6 Bertele V, Di Minno G, de Gaetano G. U-46619, a stable analogue of prostaglandin H2, induces retraction of human platelet-rich plasma clots. *Thromb Res* 1980; 18: 543–545.
7 Tanaka T, Sato R, Kurimoto T. Z-335, a new thromboxane A(2) receptor antagonist, prevents arterial thrombosis induced by ferric chloride in rats. *Europ J Pharmacol* 2000; 401: 413–418.
8 Nakamura T, Ariyoshi H, Kambayashi J et al. Signal transduction system in epinephrine stimulated platelets; comparison between epinephrine sensitive and insensitive platelets. *Thromb Res* 1997; 85: 83–93.
9 Kambayashi J, Shinoki N, Nakamura T et al. Prevalence of impaired responsiveness to epinephrine in platelets among Japanese. *Thromb Res* 1996; 81: 85–90.
10 Berndt M, Du X, Booth W. Ristocetin-dependent reconstitution of binding of von Willebrand factor to purified human platelet membrane glycoprotein Ib–IX complex. *Biochemistry* 1988; 27: 633–640.
11 Thomas K, Tune E, Choong S. Parallel determination of von Willebrand factor–Ristocetin and Botrocetin cofactors. *Thromb Res* 1994; 75: 401–408.
12 Andrews R, Booth W, Gorman J, Castaldi P, Berndt M. Purification of botrocetin from *Bothrops jararaca* venom. Analysis of the botrocetin-mediated interaction between von Willebrand factor and the human platelet membrane glycoprotein Ib-IX complex. *Biochemistry* 1989; 28: 8317–8326.
13 Quinn M, Fitzgerald D. Ticlopidine and Clopidogrel. *Circulation* 1999; 100: 1667–1672.
14 Dogne J, de Leval X, Delarge J, David J, Masereel B. New trends in thromboxane and prostacyclin modulators. *Curr Med Chem* 2000; 7: 609–628.
15 Nurden A, Poujol C, Durrieu-Jais C, Nurden P. Platelet glycoprotein IIb/IIIa inhibitors: basic and clinical aspects. *Arterioscler Thromb Vasc Biol* 1999; 19: 2835–2840.
16 Cox D, Smith R, Quinn M, Theroux P, Crean P, Fitzgerald D. Evidence of platelet activation during treatment with a GPIIb/IIIa antagonist in patients presenting with acute coronary syndromes. *J Am Coll Cardiol* 2000; 36: 1514–1519.
17 Haslam R, Dickinson N, Jang E. Cyclic nucleotides and phosphodiesterases in platelets. *Thromb Haemost* 1999; 82: 412–423.
18 Soderback U, Sollevi A. Effect of dipyridamole-like compound (R-E 244) on aggregation and cyclicAMP accumulation in human platelets. *Thromb Res* 1991; 64: 355–362.
19 Cooper J, Hill S, Alexander S, Rubin P, Horn E. Adenosine receptor-induced cyclic AMP generation and inhibition of 5-hydroxytryptamine release in human platelets. *Br J Clin Pharmacol* 1995; 40: 43–50.
20 Born G. The aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature* 1962; 194: 927.
21 Herzberg M, MacFarlane G, Gong K et al. The platelet interactivity phenotype of *Streptococcus sanguis* influences the course of experimental endocarditis. *Infec Immun* 1992; 60: 4809–4818.
22 Ford I, Douglas C, Cox D, Rees D, Heath J, Preston F. The role of immunoglobulin G and fibrinogen in platelet aggregation by *Streptococcus sanguis*. *Br J Haematol* 1997; 97: 737–746.
23 Kereiakes D, Broderick T, Roth E et al. High platelet count in platelet-rich plasma reduces measured platelet inhibition by abciximab but not tirofiban nor eptifibatide glycoprotein IIb/IIIa receptor antagonists. *J Thromb Thrombolysis* 2000; 9: 149–155.
24 Kereiakes D, Berkowitz S, Lincoff A et al. Clinical correlates and course of thrombocytopenia during percutaneous coronary intervention in the era of abciximab platelet glycoprotein IIb/IIIa blockade. *Am Heart J* 2000; 140: 74–80.
25 Glusa E, Markwardt F. Platelet functions in recombinant hirudin-anticoagulated blood. *Haemostasis* 1990; 20: 112–118.
26 Cox D, Douglas C, Preston F. The effects of anticoagulation on platelet aggregation. *Thromb Haemost* 1997; 77: 301.
27 Phillips D, Teng W, Arfsten A et al. Effect of Ca^{2+} on GP IIb–IIIa interactions with integrilin: enhanced GPIIb–IIIa binding and inhibition of platelet aggregation by reductions in the concentration of ionized calcium in plasma anticoagulated with citrate. *Circulation* 1997; 96: 1488–1494.
28 Storey R, Wilcox R, Heptinstall S. Differential effects of glycoprotein IIb/IIIa antagonists on platelet microaggregate and macroaggregate formation and effect of anticoagulant on antagonist potency. *Circulation* 1998; 98: 1616–1621.
29 Nicholson N, Panzer-Knodle S, Haas N et al. Assessment of platelet function assays. *Am Heart J* 1998; 135: S170–178.
30 Storey R, May J, Wilcox R, Heptinstall S. A whole blood assay of inhibition of platelet aggregation by glycoprotein IIb/IIIa antagonists: comparison with other aggregation methodologies. *Thromb Haemost* 1999; 82: 1307–1311.
31 Matzdorff A, Kuhnel G, Kemkes-Matthes B, Pralle H, Voss R, Fareed J. Effect of glycoprotein IIb/IIIa inhibitors on CD62p expression, platelet aggregates and microparticles in vitro. *J Laborat Clin Med* 2000; 135: 247–255.
32 Yang L, Yatomi Y, Satoh K, Ozaki Y. Inhibitory effects of bera-

Cambridge Books Online © Cambridge University Press, 2009

prost on platelet aggregation: comparative study utilizing two methods of aggregometry. *Thromb Res* 1999; 94: 25–32.
33 Carville D, Schleckser P, Guyer K, Corsello M, Walsh M. Whole blood platelet function assay on the ICHOR point-of-care hematology analyzer. *J Extra Corporal Technol* 1998; 30: 171–177.
34 Kundu S, Heilmann E, Sio R, Garcia C, Ostgaard R. Characterization of an in vitro platelet function analyzer, PFA-100TM. *Clin Appl Thromb/Hemostas* 1996; 2: 241–249.
35 Hezard N, Metz D, Nazeyrollas P et al. Use of the PFA-100 apparatus to assess platelet function in patients undergoing PTCA during and after infusion of c7E3 Fab in the presence of other antiplatelet agents. *Thromb Haemost* 2000; 83: 540–544.
36 Marshall P, Williams A, Dixon R, Growcott J, Warburton S, Armstrong J et al. A comparison of the effects of aspirin on bleeding time measured using the Simplate method and closure time measured using the PFA-100, in healthy volunteers. *Br J Clin Pharmacol* 1997; 44: 151–155.
37 Homoncik M, Jilma B, Hergovich N, Stohlawetz P, Panzer S, Speiser W. Monitoring of aspirin (ASA) pharmacodynamics with the platelet function analyzer PFA-100. *Thromb Haemost* 2000; 83: 316–321.
38 Smith J, Steinhubl S, Lincoff A, Coleman J, Lee T, Hillman R et al. Rapid platelet-function assay: an automated and quantitative cartridge-based method. *Circulation* 1999; 99: 620–625.
39 Kereiakes D, Mueller M, Howard W et al. Efficacy of Abciximab induced platelet blockade using a rapid point of care assay. *J Thromb Thrombolysis* 1999; 7: 265–275.
40 Steinhubl S, Kottke-Marchant K, Molitterno D et al. Attainment and maintenance of platelet inhibition through standard dosing of abciximab in diabetic and non-diabetic patients undergoing percutaneous coronary intervention. *Circulation* 1999; 100: 1977–1982.
41 Theroux P, Gosselin G, Nasmith J et al. The accumetrics rapid platelet function analyzer (RPFA®) to monitor platelet aggregation during oral administration of a GPIIb/IIIa antagonist. *J Am Coll Cardiol* 1999; Abstract.
42 Mascelli M, Worley S, Veriabo N et al. Rapid assessment of platelet function with a modified whole-blood aggregometer in percutaneous transluminal coronary angioplasty patients receiving anti-GP Ilb/Illa therapy. *Circulation* 1997; 96: 3860–3866.
43 Riess H, Braun G, Brehm G, Hiller E. Critical evaluation of platelet aggregation in whole human blood. *Am J Clin Pathol* 1986; 85: 50–56.
44 Gresele P, Zoja C, Deckmyn H, Arnout J, Vermylen J, Verstraete M. Dipyridamole inhibits platelet aggregation in whole blood. *Thromb Haemost* 1983; 50: 852–856.
45 deLa Cruz J, Romero M, Sanchex P, Sanchez de La Cuesta F. Antiplatelet effect of pentoxyfylline in human whole blood. *Gen Pharmacol* 1993; 24: 605–609.
46 Podczasy J, Lee J, Vucenik I. Evaluation of whole-blood lumi-aggregation. *Clin Appl Thromb/Hemost* 1997; 3: 190–195.
47 Harder S, Kirchmaier C, Krzywanek H, Westrup D, Bae J, Breddin H. Pharmacokinetics and pharmacodynamic effects of a new antibody glycoprotein IIb/IIIa inhibitor (YM337) in healthy subjects. *Circulation* 1999; 100: 1175–1181.
48 Schneider D, Taatjes D, Sobel B. Paradoxical inhibition of fibrinogen binding and potentiation of alpha-granule release by specific types of inhibitors of glycoprotein IIb–IIIa. *Cardiovasc Res* 2000; 45: 437–446.
49 Zamarron C, Ginsberg M, Plow E. A receptor-induced binding site in fibrinogen elicited by its interaction with platelet membrane glycoprotein IIb–IIIa. *J Biol Chem* 1991; 266: 16193–16199.
50 Ugarova T, Budzynski A, Shattil S, Ruggeri Z, Ginsberg M, Plow E. Conformational changes in fibrinogen elicited by its interaction with platelet membrane glycoprotein GPIIb–IIIa. *J Biol Chem* 1993; 268: 21080–21087.
51 Bihour C, Durrieu-Jais C, Besse P, Nurden P, Nurden A. Flow cytometry reveals activated GP IIb-IIIa complexes on platelets from patients undergoing thrombolytic therapy after acute myocardial infarction. *Blood Coagul Fibrinol* 1995; 6: 395–410.
52 Shattil S, Hoxie J, Cunningham M, Brass L. Changes in the platelet membrane glycoprotein IIb.IIIa complex during platelet activation. *J Biol Chem* 1985; 260: 11107–11114.
53 Moshfegh K, Redondo M, Julmy F et al. Antiplatelet effects of clopidogrel compared with aspirin after myocardial infarction: enhanced inhibitory effects of combination therapy. *J Am Coll Cardiol* 2000; 36: 699–705.
54 Du X, Plow E, Frelinger A, O'Toole T, Loftus J, Ginsberg M. Ligands 'activate' integrin alpha IIb beta 3 (platelet GPIIb–IIIa). *Cell* 1991; 65: 409–416.
55 Mondoro T, Wall C, White M, Jennings L. Selective induction of a glycoprotein IIIa ligand-induced binding site by fibrinogen and von Willebrand factor. *Blood* 1996; 88: 3824–3830.
56 Nurden P, Humbert M, Piotrowicz R et al. Distribution of ligand-occupied alpha IIb beta 3 in resting and activated human platelets determined by expression of a novel class of ligand-induced binding site recognized by monoclonal antibody AP6. *Blood* 1996; 88: 887–899.
57 Murphy N, Pratico D, Fitzgerald D. Functional relevance of the expression of ligand-induced binding sites in the response to platelet GP IIb/IIIa antagonists in vivo. *J Pharmacol Exp Therap* 1998; 286: 945–951.
58 Giugliano R, McCabe C, Sequeira R et al. First report of an intravenous and oral glycoprotein IIb/IIIa inhibitor (RPR 109891) in patients with recent acute coronary syndromes: results of the TIMI 15A and 15B trials. *Am Heart J* 2000; 140: 81–93.
59 Jennings L, Haga J, Slack S. Differential expression of a ligand induced binding site (LIBS) by GPIIb–IIIa ligand recognition peptides and parenteral antagonists. *Thromb Haemost* 2000; 84: 1095–1102.
60 Quinn M, Deering A, Stewart M, Cox D, Foley B, Fitzgerald D. Quantifying GPIIb/IIIa receptor binding using 2 monoclonal antibodies: discriminating abciximab and small molecular weight antagonists. *Circulation* 1999; 99: 2231–2238.
61 Quinn M, Cox D, Foley J, Fitzgerald D. Glycoprotein IIb/IIIa

Cambridge Books Online © Cambridge University Press, 2009

receptor number and occupancy during chronic administration of an oral antagonist. *J Pharmacol Exp Therap* 2000; 295: 670–676.
62 Hezard N, Metz D, Nazeyrollas P et al. Free and total platelet glycoprotein IIb/IIIa measurement in whole blood by quantitative flow cytometry during and after infusion of c7E3 Fab in patients undergoing PTCA. *Thromb Haemost* 1999; 81: 869–873.
63 Ciabattoni G, Maclouf J, Catella F, FitzGerald G, Patrono C. Radioimmunoassay of 11-dehydrothromboxane B2 in human plasma and urine. *Biochim Biophys Acta* 1987; 918: 293–297.
64 Feldman M, Cryer B. Aspirin absorption rates and platelet inhibition times with 325-mg buffered aspirin tablets (chewed or swallowed intact) and with buffered aspirin solution. *Am J Cardiol* 1999; 84: 404–409.
65 Brown N, May J, Wilcox R et al. Comparison of antiplatelet activity of microencapsulated aspirin 162.5 Mg (Caspac XL), with enteric coated aspirin 75 mg and 150 mg in patients with atherosclerosis. *Br J Clin Pharmacol* 1999; 48: 57–62.
66 Belton O, Byrne D, Kearney D, Leahy A, Fitzgerald D. Cyclooxygenase-1 and -2-dependent prostacyclin formation in patients with atherosclerosis. *Circulation* 2000; 102: 840–845.
67 Ciabattoni G, Boss A, Daffonchio L et al. Radioimmunoassay measurement of 2,3-dinor metabolites of prostacyclin and thromboxane in human urine. *Adv Prostaglandin Thromboxane Leukotriene Res* 1987; 17: 598–602.
68 Bode-Boger S, Boger R, Schubert M, Frolich J. Effects of very low dose and enteric-coated acetylsalicylic acid on prostacyclin and thromboxane formation and on bleeding time in healthy subjects. *Eur J Clin Pharmacol* 1998; 54: 707–714.
69 Bode-Boger S, Boger R, Kienke S et al. Chronic dietary supplementation with L-arginine inhibits platelet aggregation and thromboxane A2 synthesis in hypercholesterolaemic rabbits in vivo. *Cardiovasc Res* 1998; 37: 756–764.
70 Van Hecken A, Schwartz J, Depre M et al. Comparative inhibitory activity of rofecoxib, meloxicam, diclofenac, ibuprofen, and naproxen on COX-2 versus COX-1 in healthy volunteers. *J Clin Pharmacol* 2000; 40: 1109–1120.
71 Perneby C, Granstrom E, Beck O, Fitzgerald D, Harhen B, Hjemdahl P. Optimization of an enzyme immunoassay for 11-dehydro-thromboxane B(2) in urine: comparison with GC-MS. *Thromb Res* 1999; 96: 427–436.
72 Kawasaki T, Ozeki Y, Igawa T, Kambayashi J. Increased platelet sensitivity to collagen in individuals resistant to low-dose aspirin. *Stroke* 2000; 31: 591–595.
73 Fujitani K, Kambayashi J, Murata K et al. Clinical evaluation on combined administration of oral prostacyclin analogue beraprost and phosphodiesterase inhibitor cilostazol. *Pharmacol Res* 1996; 31: 121–125.
74 Gries A, Bode C, Peter K et al. Inhaled nitric oxide inhibits human platelet aggregation, P-selectin expression, and fibrinogen binding in vitro and in vivo. *Circulation* 1998; 97: 1481–1487.
75 Zhdanova I, Raz D. Effects of melatonin ingestion on cAMP and cGMP levels in human plasma. *J Endocrinol* 1999; 163: 457–462.
76 Albert J, Wallen N, Li N, Frostell C, Hjemdahl P. Neither endogenous nor inhaled nitric oxide influences the function of circulating platelets in healthy volunteers. *Clin Sci* 1999; 97: 345–353.
77 Cox D, Aoki T, Seki J, Motoyama Y, Yoshida K. Pentamidine is a specific, non-peptide, GPIIb/IIIa antagonist. *Thromb Haemost* 1996; 75: 503–509.
78 Goto S, Salomon D, Ikeda Y, Ruggeri Z. Characterization of the unique mechanism mediating the shear-dependent binding of soluble von Willebrand factor to platelets. *J Biol Chem* 1995; 270: 23352–23361.
79 Ruggeri Z. The role of von Willebrand factor and fibrinogen in the initiation of platelet adhesion to thrombogenic surfaces. *Thromb Haemost* 1995; 74: 460–463.
80 Sixma J, de Groot P, van Zanten H, IJsseldijk M. A new perfusion chamber to detect platelet adhesion using a small volume of blood. *Thromb Res* 1998; 92: S43–S46.
81 Varon D, Lashevski I, Brenner B et al. Cone and plate(let) analyzer: monitoring glycoprotein IIb/IIIa antagonists and von Willebrand disease replacement therapy by testing platelet deposition under flow conditions. *Am Heart J* 1998; 135: S187–S193.
82 Savage B, Ginsberg M, Ruggeri Z. Influence of fibrillar collagen structure on the mechanisms of platelet thrombus formation under flow. *Blood* 1999; 94: 2704–2715.
83 Schenk J, Radziwon P, Morsdorf S, Eckenberger P, Breddin H. Effects of aprosulate, a novel synthetic glycosaminoglycan, on coagulation and platelet function parameters: a prospective, randomized phase I study. *Clin Appl Thromb/Hemost* 1999; 5: 192–197.
84 Fredrickson B, Turner N, Kleiman N et al. Effects of abciximab, ticlopidine, and combined Abciximab/Ticlopidine therapy on platelet and leukocyte function in patients undergoing coronary angioplasty. *Circulation* 2000; 101: 1122–1129.
85 Kamat S, Turner N, Konstantopoulos K et al. Effects of Integrelin on platelet function in flow models of arterial thrombosis. *J Cardiovasc Pharmacol* 1997; 29: 156–163.
86 Sramek R, Sramek A, Koster T, Briet E, Rosendaal F. A randomized and blinded comparison of three bleeding time techniques: the Ivy method, and the Simplate II method in two directions. *Thromb Haemost* 1992; 67: 514–518.
87 Simoons M, de Boer M, van den Brand M et al. Randomized trial of a GPIIb/IIIa platelet receptor blocker in refractory unstable angina. *Circulation* 1994; 89: 596–603.
88 Roux S, Bunting S, Kouns W, Steiner B. Overdosing of potent GPIIb/IIIa antagonists differentially prolongs the bleeding time in guinea pigs. *Thromb Haemost* 1993; 69: 560.
89 Boneu B, Destelle G. Platelet anti-aggregating activity and tolerance of clopidogrel in atherosclerotic patients. *Thromb Haemost* 1996; 76: 939–943.
90 Pogliani E, Fowst C, Bregani R, Corneo G. Bleeding time and antiplatelet agents in normal volunteers. *Int J Clin Laborat Res* 1992; 22: 58–61.

Cambridge Books Online © Cambridge University Press, 2009

91 Warning A, Nies D, Wolf H, Welzel D. Effect of low molecular weight heparin on coagulation parameters and bleeding time in healthy volunteers. *Arzneimittelforschung* 1987; 37: 847–849.

92 Bernardi M, Califf R, Kleiman N, Ellis S, Topol E. Lack of usefulness of prolonged bleeding times in predicting hemorrhagic events in patients receiving the 7E3 glycoprotein IIb/IIIa platelet antibody. The TAMI Study Group. *Am J Cardiol* 1993; 72: 1121–1125.

93 De Caterina R, Lanza M, Manca G, Strata G, Maffei S, Salvatore L. Bleeding time and bleeding: an analysis of the relationship of the bleeding time test with parameters of surgical bleeding. *Blood* 1994; 84: 3363–3370.

94 Mascelli M, Lance E, Damaraju L, Wagner C, Weisman H, Jordan R. Pharmacodynamic profile of short-term abciximab treatment demonstrates prolonged platelet inhibition with gradual recovery from GP IIb/IIIa receptor blockade. *Circulation* 1998; 97: 1680–1688.

95 Rosenkranz B, Frolich J. Plasma concentrations and antiplatelet effects after low dose acetylsalicylic acid. *Prostaglandins Leukotriene Med* 1985; 19: 289–300.

96 Solum N. Procoagulant expression in platelets and defects leading to clinical disorders. *Arterioscler Thromb Vasc Biol* 1999; 19: 2841–2846.

97 Dangas G, Marmur J, King T et al. Effects of platelet glycoprotein IIb/IIIa inhibition with abciximab on thrombin generation and activity during percutaneous coronary intervention. *Am Heart J* 1999; 138: 49–54.

98 Coiffic A, Cazes E, Janvier G, Forestier F, Lanza F, Nurden A et al. Inhibition of platelet aggregation by abciximab but not by aspirin can be detected by a new point-of-care test, the hemostatus. *Thromb Res* 1999; 95: 83–91.

99 Li C, Hoffmann T, Hsieh P, Malik S, Watson W. The Xylum Clot Signature Analyzer: a dynamic flow system that simulates vascular injury. *Thromb Res* 1998; 92: S67–S77.

100 Igawa T, Kornhauser R, Cilla D, King J, Kambayashi J. Evaluation of the Clot Signature Analyzer as a hemostasis test in healthy volunteers exposed to low doses of aspirin. *Clin Appl Thromb/Hemost* 1999; 5: 117–121.

101 Miyashita T, Kuro M. Evaluation of platelet function by Sonoclot analysis compared with other hemostatic variables in cardiac surgery. *Anesth Analges* 1998; 87: 1228–1233.

102 LaForce W, Brudno D, Kanto W, Karp W. Evaluation of the SonoClot Analyzer for the measurement of platelet function in whole blood. *Ann Clin Laborat Sci* 1992; 22: 30–33.

103 Rooney M, Farrell D, van Hemel B, de Groot P, Lord S. The contribution of the three hypothesized integrin-binding sites in fibrinogen to platelet-mediated clot retraction. *Blood* 1998; 92: 2374–2381.

104 Mousa S, Khurana S, Forsythe M. Comparative in vitro efficacy of different platelet glycoprotein IIb/IIIa antagonists on platelet-mediated clot strength induced by tissue factor with use of thromboelastography: differentiation among glycoprotein IIb/IIIa antagonists. *Arterioscler Thromb Vasc Biol* 2000; 20: 1162–1167.

105 Carr M. Measurement of platelet force: the hemodyne hemostasis analyzer. *Clin Laborat Managem Rev* 1995; 9: 312–318.

106 Greilich P, Alving B, Longnecker D et al. Near-site monitoring of the antiplatelet drug abciximab using the Hemodyne analyzer and modified thrombelastograph. *J Cardiothoracic Vasc Anesthesia* 1999; 13: 58–64.

107 Putter M, Grotemeyer K, Wurthwein G et al. Inhibition of smoking-induced platelet aggregation by aspirin and pycnogenol. *Thromb Res* 1999; 95: 155–161.

108 Fusegawa Y, Goto S, Handa S, Kawada T, Ando Y. Platelet spontaneous aggregation in platelet-rich plasma is increased in habitual smokers. *Thromb Res* 1999; 93: 271–278.

109 Pernerstorfer T, Stohlawetz P, Stummvoll G et al. Low-dose aspirin does not lower in vivo platelet activation in healthy smokers. *Br J Haematol* 1998; 102: 1229–1231.

110 Law M, Morris J, Wald N. Environmental tobacco smoke exposure and ischaemic heart disease: an evaluation of the evidence. *Br Med J* 1997; 315: 973–980.

111 Smith C, Fischer T, Sears S. Environmental tobacco smoke, cardiovascular disease, and the nonlinear dose-response hypothesis. *Toxicol Sci* 2000; 54: 462–472.

112 Chiabrando C, Rivoltella L, Alberti E, Bonollo M, Djurup R, Fanelli R. Urinary excretion and origin of 11-dehydro-2,3-dinor-thromboxane B2 in man. *Prostaglandins* 1993; 45: 401–411.

113 Roy S. Effects of smoking on prostacyclin formation and platelet aggregation in users of oral contraceptives. *Am J Obst Gyn* 1999; 180: S364–S368.

114 Weber A, Liesener S, Schanz A, Hohlfeld T, Schror K. Habitual smoking causes an abnormality in platelet thromboxane A2 metabolism and results in an altered susceptibility to aspirin effects. *Platelets* 2000; 11: 177–182.

115 Durand P, Prost M, Blache D. Folic acid deficiency enhances oral contraceptive-induced platelet hyperactivity. *Arterioscler Thromb Vasc Biol* 1997; 17: 1939–1946.

116 Mezzano D, Kosiel K, Martinez C et al. Cardiovascular risk factors in vegetarians. Normalization Of hyperhomocysteinemia with vitamin B(12) and reduction of platelet aggregation with n-3 fatty acids. *Thromb Res* 2000; 100: 153–160.

117 Cipollone F, Ciabattoni G, Patrignani P et al. Oxidant stress and aspirin-insensitive thromboxane biosynthesis in severe unstable angina. *Circulation* 2000; 102: 1007–1013.

118 Wilkinson I, Megson I, MacCallum H, Sogo N, Cockcroft J, Webb D. Oral vitamin C reduces arterial stiffness and platelet aggregation in humans. *J Cardiovasc Pharmacol* 1999; 34: 690–693.

119 Pignatelli P, Pulcinelli F, Lenti L, Gazzaniga P, Violi F. Vitamin E inhibits collagen-induced platelet activation by blunting hydrogen peroxide. *Arterioscler Thromb Vasc Biol* 1999; 19: 2542–2547.

120 Chang S, Chuang H, Chen H. Vitamin B6 down-regulates the expression of human GPIIb gene. *J Nutrit Sci Vitaminol (Tokyo)* 1999; 45: 471–479.

121 Kabakus N, Yilmaz B, Caliskan U. Investigation of platelet aggregation by impedance and optic methods in children

Cambridge Books Online © Cambridge University Press, 2009

with iron deficiency anaemia. *Haematologia (Budap)* 2000; 30: 107–115.

122 Kurekci A, Atay A, Sarici S, Zeybek C, Koseoglu V, Ozcan O. Effect of iron therapy on the whole blood platelet aggregation in infants with iron deficiency anemia. *Thromb Res* 2000; 97: 281–285.

123 Nguyen A, Packham M, Rand M. Effects of ethanol on platelet responses associated with adhesion to collagen. *Thromb Res* 1999; 95: 303–314.

124 Zhang Q, Das K, Siddiqui S, Myers A. Effects of acute, moderate ethanol consumption on human platelet aggregation in platelet-rich plasma and whole blood. *Alcohol Clin Exp Res* 2000; 24: 528–534.

125 Ali M, Bordia T, Mustafa T. Effect of raw versus boiled aqueous extract of garlic and onion on platelet aggregation. *Prostaglandins Leukotriene Med* 1999; 60: 43–47.

126 Mori T, Beilin L, Burke V, Morris J, Ritchie J. Interactions between dietary fat, fish, and fish oils and their effects on platelet function in men at risk of cardiovascular disease. *Arterioscler Thromb Vasc Biol* 1997; 17: 279–286.

127 Varani K, Portaluppi F, Gessi S et al. Dose and time effects of caffeine intake on human platelet adenosine A(2A) receptors: functional and biochemical aspects. *Circulation* 2000; 102: 285–289.

128 Li N, Wallen N, Hjemdahl P. Evidence for prothrombotic effects of exercise and limited protection by aspirin. *Circulation* 1999; 100: 1374–1379.

129 Hurlen M, Seljeflot I, Arnesen H. Increased platelet aggregability during exercise in patients with previous myocardial infarction. Lack of inhibition by aspirin. *Thromb Res* 2000; 99: 487–494.

130 Hsu H, Lee Y, Chen M. Exercise shifts the platelet aggregation modulatory role from native to mildly oxidized low-density lipoprotein. *Med Sci Sports Exercise* 2000; 32: 933–939.

131 Feng D, Murillo J, Jadhav P et al. Upright posture and maximal exercise increase platelet aggregability and prostacyclin production in healthy male subjects. *Br J Sports Med* 1999; 33: 401–404.

132 Lindemann S, Klingel B, Fisch A, Meyer J, Darius H. Increased platelet sensitivity toward platelet inhibitors during physical exercise in patients with coronary artery disease. *Thromb Res* 1999; 93: 51–59.

133 Faraday N, Goldschmidt-Clermont P, Bray P. Gender differences in platelet GPIIb-IIIa activation. *Thromb Haemost* 1997; 77: 748–754.

134 Bar J, Lahav J, Hod M, Ben-Rafael Z, Weinberger I, Brosens J. Regulation of platelet aggregation and adenosine triphosphate release in vitro by 17beta-estradiol and medroxyprogesterone acetate in postmenopausal women. *Thromb Haemost* 2000; 84: 695–700.

135 Boudoulas K, Cooke G, Roos C, Bray P, Goldschmidt-Clermont P. The PlA polymorphism of glycoprotein IIIa functions as a modifier for the effect of estrogen on platelet aggregation. *Arch Path Laborat Med* 2001; 125: 112–115.

136 Musselman D, Tomer A, Manatunga A et al. Exaggerated platelet reactivity in major depression. *Am J Psychiat* 1996; 153: 1313–1317.

137 Musselman D, Marzec U, Manatunga A et al. Platelet reactivity in depressed patients treated with paroxetine: preliminary findings. *Arch Gen Psychiat* 2000; 57: 875–882.

138 Bray P. Platelet glycoprotein polymorphisms as risk factors for thrombosis. *Curr Opin Hematol* 2000; 7: 284–289.

139 Feng D, Lindpaintner K, Larson M et al. Increased platelet aggregability associated with platelet GPIIIa PlA2 polymorphism: the Framingham Offspring Study. *Arterioscler Thromb Vasc Biol* 1999; 19: 1142–1147.

140 Michelson A, Furman M, Goldschmidt-Clermont P et al. Platelet GP IIIa Pl(A) polymorphisms display different sensitivities to agonists. *Circulation* 2000; 101: 1013–1018.

141 Corral J, Gonzalez-Conejero R, Rivera J, Iniesta J, Lozano M, Vicente V. HPA-1 genotype in arterial thrombosis—role of HPA-1b polymorphism in platelet function. *Blood Coagul Fibrinol* 1997; 8: 284–290.

142 O'Connor F, Molloy M, Shields D, Fitzgerald DJ. The influence of the platelet PLA2 polymorphism on the response to a glycoprotein IIb/IIIa antagonist in patients with acute coronary syndromes. *Blood* 1999; 94: 244a.

143 Szczeklik A, Undas A, Sanak M, Frolow M, Wegrzyn W. Short report: relationship between bleeding time, aspirin and the PlA1/A2 polymorphism of platelet glycoprotein IIIa. *Br J Haematol* 2000; 110: 965–967.

144 Kritzik M, Savage B, Nugent D, Santoso S, Ruggeri Z, Kunicki T. Nucleotide polymorphisms in the alpha2 gene define multiple alleles that are associated with differences in platelet alpha2 beta1 density. *Blood* 1998; 92: 2382–2388.

145 Afshar-Kharghan V, Li C, Khoshnevis-Asl M, Lopez J. Kozak sequence polymorphism of the glycoprotein (GP) Ibalpha gene is a major determinant of the plasma membrane levels of the platelet GPIb–IX–V complex. *Blood* 1999; 94: 186–191.

146 Croft S, Hampton K, Daly M, Steeds R, Channer K, Samani N. Kozak sequence polymorphism in the platelet GPIbalpha gene is not associated with risk of myocardial infarction. *Blood* 2000; 95: 2183–2184.

Cambridge Books Online © Cambridge University Press, 2009

32

Flow cytometric analysis of platelet function

Alan D. Michelson, Marc R. Barnard, Lori A. Krueger, A.L. Frelinger III and Mark I. Furman

Center for Platelet Function Studies and Division of Cardiovascular Medicine, University of Massachusetts Medical School, Worcester, USA

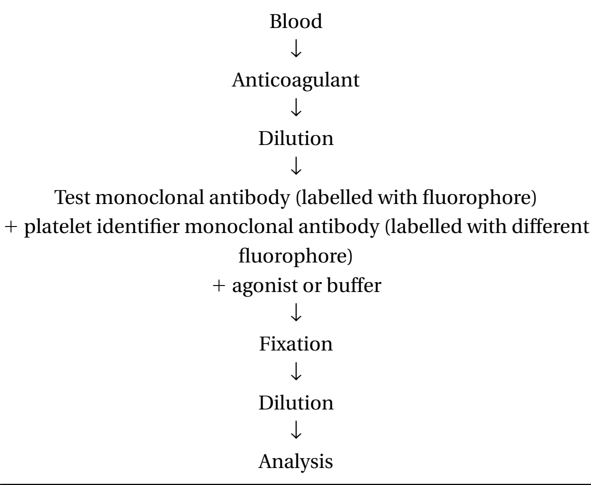
Introduction

This chapter will review the use of flow cytometry for the detection of circulating activated platelets and the analysis of many other aspects of platelet function[1,2]. Flow cytometry rapidly measures the specific characteristics of a large number of individual cells. Before flow cytometric analysis, cells in suspension are fluorescently labelled, typically with a fluorescently conjugated monoclonal antibody. In the flow cytometer, the suspended cells pass through a flow chamber and, at a rate of 1 000–10 000 cells per minute, through the focused beam of a laser. After fluorescent activation of the fluorophore at the excitation wavelength, a detector processes the emitted fluorescence and light scattering properties of each cell. (Ref.[3] provides a very readable overview of the principles of flow cytometry.)

In the absence of an added exogenous platelet agonist, whole blood flow cytometry can determine the activation state of circulating platelets, as judged by the binding of an activation-dependent monoclonal antibody. In addition to this assessment of platelet function in vivo, inclusion of an exogenous agonist in the assay enables analysis of the reactivity of circulating platelets in vitro. In the latter application, whole blood flow cytometry is a physiological assay of platelet function in that an agonist results in a specific functional response by the platelets: a change in the surface expression of a physiological receptor (or other antigen or bound ligand), as determined by a change in the binding of a monoclonal antibody.

A typical schema of sample preparation for whole blood flow cytometry is shown in Table 32.1. The anticoagulant is usually buffered sodium citrate, although other anticoagulants can be used[4]. The purpose of the initial dilution is to minimize the formation of platelet aggregates (see below). Typically, two monoclonal antibodies are used, each labelled with a different fluorophore. A wide variety of fluorophores are available for antibody conjugation, e.g. phycoerythrin, fluorescein isothiocyanate (FITC), peridinin chlorophyll protein (PerCP), phycoerythrin-Cy5, phycoerythrin-Texas Red, allophycocyanin (APC). The 'test' monoclonal antibody (recognizing the antigen to be measured) should be added in a saturating concentration. The 'platelet identifier' monoclonal antibody, e.g. glycoprotein (GP) Ib-, IIb-, or IIIa-specific should be added at a near saturating concentration. Physiological agonists that can be used in the assay include thrombin, thrombin receptor-activating peptide (TRAP), ADP, collagen, and thromboxane A_2 analogues. Samples are stabilized by fixation, typically with a final concentration of 1% paraformaldehyde. The test monoclonal antibody can be added after fixation, provided fixation does not interfere with antibody binding[5] (see below).

Table 32.1. A typical schema of sample preparation for analysis of platelets by whole blood flow cytometry



Blood
↓
Anticoagulant
↓
Dilution
↓
Test monoclonal antibody (labelled with fluorophore)
+ platelet identifier monoclonal antibody (labelled with different fluorophore)
+ agonist or buffer
↓
Fixation
↓
Dilution
↓
Analysis

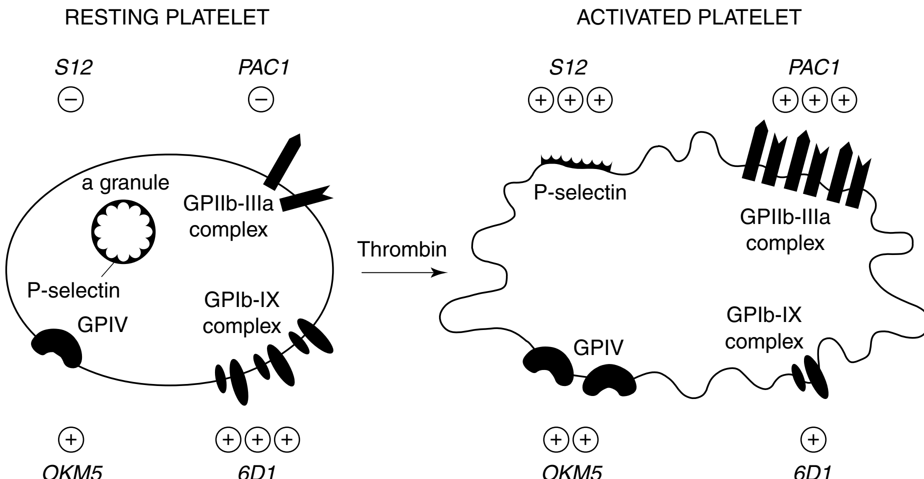


Fig. 32.1. Effect of platelet activation on monoclonal antibody binding. The cartoon depicts the binding of monoclonal antibodies (*in italics*) to resting platelets and the relative change in the binding of these antibodies after thrombin activation. *S12* is directed at the α granule membrane protein P-selectin (CD62P). P-selectin is not detectable on the surface of resting platelets. After thrombin activation, P-selectin is translocated to the platelet plasma membrane. Thus, *S12* only binds to the surface of activated platelets. *PAC1* is directed at the fibrinogen binding site on the GPIIb–IIIa complex. This fibrinogen binding site is not exposed on resting platelets. Thrombin stimulation results in a conformational change in the GPIIb–IIIa complex that exposes the fibrinogen binding site. Thus, *PAC1* only binds to the surface of activated platelets. *OKM5* is directed at the thrombospondin binding site on GPIV. *OKM5* binds to resting platelets but binding is increased following thrombin stimulation. *6D1* is directed at the von Willebrand factor binding site on GPIb. In contrast to the other monoclonal antibodies, the binding of *6D1* is markedly reduced following thrombin stimulation. (Modified[28]).

Samples are then analyzed in a flow cytometer. After identification of platelets both by their characteristic light scatter and by, for example, phycoerythrin positivity, binding of the, for example, FITC-conjugated test monoclonal antibody is determined by analysing 5000–10000 individual platelets for phycoerythrin fluorescence. Background binding obtained from parallel samples with FITC-conjugated, isotypic species-specific immunoglobulin may be subtracted from each test sample.

Monoclonal antibodies

Laboratory markers of platelet activation include activation-dependent changes in the glycoprotein GPIIb–IIIa complex, exposure of granule membrane proteins, binding of secreted platelet proteins, and development of a procoagulant surface (Fig. 32.1, Table 32.2).

The two most widely studied types of activation-dependent monoclonal antibodies are those directed against conformational changes in the GPIIb–IIIa complex and those directed against granule membrane proteins (Fig. 32.1, Table 32.2). The GPIIb–IIIa complex (CD41/CD61, integrin $\alpha_{IIb}\beta_3$) is a receptor for fibrinogen, von Willebrand factor, fibronectin, and vitronectin that is essential for platelet aggregation[6]. Whereas most monoclonal antibodies directed against the GPIIb–IIIa complex bind to resting platelets, monoclonal antibody PAC1 is directed against the fibrinogen binding site exposed by a conformational change in the GPIIb–IIIa complex of activated platelets (Fig. 32.1)[7]. Thus, PAC1 only binds to activated platelets, not to resting platelets. Other GPIIb–IIIa-specific activation-dependent monoclonal antibodies are directed against either ligand-induced conformational changes in the GPIIb–IIIa complex ('ligand-induced binding sites' or 'LIBS')[8] or receptor-induced conformational changes in the bound ligand (fibrinogen) ('receptor-induced binding sites' or 'RIBS')[9] (Table 32.2).

The most widely studied type of activation-dependent monoclonal antibodies directed against granule membrane proteins are those directed against P-selectin (CD62P). P-selectin mediates adhesion of activated platelets to monocytes and neutrophils[10]. P-selectin is a component of the α- granule membrane of resting platelets that is only expressed on the platelet surface membrane after α-granule secretion (Fig. 32.1)[11]. Therefore a P-selectin-specific monoclonal antibody only binds to degranulated platelets, not to resting platelets. The activation-dependent increase in platelet surface P-selectin is not reversible over time in vitro[12]. However, in vivo circulating degranulated

Cambridge Books Online © Cambridge University Press, 2009

Table 32.2. Activation-dependent monoclonal antibodies, i.e. antibodies that bind to activated but not resting platelets

Activation-dependent surface change	Prototypic antibodies	References
Changes in GPIIb–IIIa		
Activation-induced conformational change in GPIIb–IIIa resulting in exposure of the fibrinogen binding site	PAC1	7
Ligand-induced conformational change in GPIIb–IIIa	PM 1.1.; LIBS1; LIBS6	8,44,98
Receptor-induced conformational change in bound ligand (fibrinogen)	2G5; 9F9; F26	9,38,138
Exposure of granule membrane proteins		
P-selectin (α-granules)	S12; AC1.2	72,139
CD63 (lysosomes)	CLB-gran/12	140
LAMP-1 (lysosomes)	H5G11	141
CD154	TRAP1	46
Binding of secreted platelet proteins		
Thrombospondin	P8; TSP-1	142,143
Multimerin	JS-1	144,145
Development of a procoagulant surface		
Factor Va binding	V237	108
Factor VIII binding	1B3	109

Source: Modified from Michelson and Shattil[146].

platelets rapidly lose their surface P-selectin, but continue to circulate and function[13,14]. Platelet surface P-selectin is therefore not an ideal marker for the detection of circulating degranulated platelets, unless (i) the blood sample is drawn immediately distal to the site of platelet activation, (ii) the blood sample is drawn within 5 minutes of the activating stimulus, or (iii) there is continuous activation of platelets. The length of time that other activation-dependent surface markers remain expressed on the platelet surface in vivo has not yet been determined.

Methodological issues

Advantages of flow cytometry

Other tests used to study platelet activation and function in clinical settings have limitations. Platelet aggregometry may show whether a particular clinical condition results in changes in platelet reactivity, but cannot determine whether the condition directly activates platelets[15]. In contrast, plasma assays of β-thromboglobulin[16,17], platelet factor 4[16,17], and soluble P-selectin[18], as well as plasma and urinary assays of thromboxane A_2 metabolites[19,20], may indirectly determine that a clinical condition activates platelets, but cannot measure changes in platelet reactivity associated with the condition. None of these assays can measure the extent of activation of individual platelets nor can they detect distinct subpopulations of platelets. Platelet aggregation studies are semiquantitative and subject to standardization problems[15,21]. As a result of the plasma separation procedures required, assays of plasma β-thromboglobulin and platelet factor 4 concentrations are particularly vulnerable to artefactual in vitro platelet activation[16,17]. Furthermore, soluble P-selectin in plasma may be of endothelial cell origin[18].

Whole blood flow cytometric assays of platelet activation have none of these limitations. Platelets are directly analysed in their physiological milieu of whole blood (including red cells and white cells, both of which affect platelet activation[22,23]). The minimal manipulation of the samples prevents artefactual in vitro activation and potential loss of platelet subpopulations[24–27]. Both the activation state of circulating platelets and the reactivity of circulating platelets can be determined. The flow cytometric method permits the detection of a spectrum of specific activation-dependent modifications in the platelet surface membrane. Furthermore, as new monoclonal antibodies directed against novel functional epitopes are developed, they can easily be incorporated into the assay. A subpopulation of as

Cambridge Books Online © Cambridge University Press, 2009

few as 1% partially activated platelets can be detected by whole blood flow cytometry[28]. Only minuscule volumes (~5 μl) of blood are required[24,25] making whole blood flow cytometry particularly advantageous for neonatal studies[29]. The platelets of patients with profound thrombocytopenia can also be accurately analysed.

Platelet activation by thrombin, one of the most physiologically important platelet activators[30–32] can be directly measured in whole blood through the use of the synthetic tetrapeptide glycyl-L-prolyl-L-arginyl-L-proline (GPRP)[25,27,28]. In the absence of GPRP, addition of thrombin to whole blood results in a fibrin clot, thereby precluding the use of thrombin as an agonist in the whole blood assay. Furthermore, thrombin is a potent inducer of platelet-to-platelet aggregation, which precludes analysis by flow cytometry of activation-dependent changes in individual platelets. However, addition to whole blood of GPRP together with thrombin inhibits both fibrin polymerization and platelet-to-platelet aggregation, without affecting thrombin-induced platelet activation[25,27,28]. An alternative to the use of thrombin and GPRP in the whole blood flow cytometric assay is the use of a thrombin receptor agonist peptide (TRAP). TRAP is a peptide fragment of the protease-activated receptor 1 (PAR1) 'tethered ligand' receptor for thrombin[33]. Without the need for GPRP, TRAP directly activates platelets in whole blood without resulting in a fibrin clot[34]. However, TRAP may not reflect all aspects of thrombin-induced platelet activation, because PAR1 is not the only platelet receptor for thrombin[35,36]. Other platelet receptors for thrombin include PAR4 and GPIb[35,36].

Disadvantages of flow cytometry

There are disadvantages to flow cytometry. First, flow cytometers are expensive instruments to purchase and maintain. Secondly, for a clinical assay, sample preparation is quite complicated and requires a dedicated operator, although the future development of automated systems and more user-friendly software should simplify the assay. Thirdly, to avoid ex vivo platelet activation, blood samples should be processed within approximately 45 minutes of drawing[24]. (For some monoclonal antibodies, this problem can be circumvented by immediate fixation – see **'Fixation'** below.) Fourthly, flow cytometry only measures the function of circulating platelets, whereas plasma assays of β-thromboglobulin and platelet factor 4, and plasma and urinary assays of thromboxane A_2 metabolites, also reflect platelet activation at the blood vessel wall and recently cleared platelets. Thus, if the activated platelets are rapidly cleared, adherent to blood vessel walls or to extracorporeal circuits, or have lost surface P-selectin[13], flow cytometry may not detect evidence of platelet activation. For example, during cardiopulmonary bypass, flow cytometry demonstrates only modest evidence of circulating activated platelets[37,38], whereas radioimmunoassays of plasma β-thromboglobulin and platelet factor 4 consistently provide evidence of marked activation of platelets[39,40].

Table 32.3. Methods of sample preparation for whole blood flow cytometry that minimize the formation of platelet aggregates

Prepare reagents in advance and avoid delays in procedure
To collect blood, use a light tourniquet and a needle not narrower than 21 gauge
Smooth, easy flow from blood draw
Discard the first 2 ml of blood
Polypropylene (or siliconized glass) tubes or syringes
Immediate mixture with anticoagulant
No washing, centrifugation, gel filtration, vortexing, or stirring steps
Reduce the platelet count by dilution of the samples
If thrombin is the agonist, inclusion of the peptide GPRP in the assay
Mix gently after addition of agonist, then incubate undisturbed
Fixation

Blood drawing

After discarding the first 2 ml of blood, collection of blood into a 3.2% sodium citrate Vacutainer (Becton Dickinson, Rutherford, NJ) does not result in significant platelet activation[37]. However, each laboratory should determine whether their method of collection, including the drawing of samples through angioplasty and other catheters, results in artefactual in vitro platelet activation, as determined by the binding of activation-dependent monoclonal antibodies.

Minimizing platelet aggregates

Platelet aggregates can be measured by flow cytometry[41]. However, if the platelets are aggregated, the amount of antigen per platelet cannot be determined by flow cytometry. This is because flow cytometry measures the amount of fluorescence per individual particle, irrespective of whether the particle is a single platelet or an aggregate of an unknown number of platelets. Platelet aggregates can be minimized in the preparation of plate-

Cambridge Books Online © Cambridge University Press, 2009

lets for whole blood flow cytometry by a combination of the methods shown in Table 32.3. RGD-containing peptides can also be used to minimize platelet aggregates[42,43], but these peptides may interfere with the binding of detecting antibodies, e.g. PAC1 and result in exposure of LIBS[8,44]. Each sample should be monitored for evidence of platelet aggregation ('smearing' of the platelets into the upper right quadrant of the log side (orthogonal) light scatter vs. log forward light scatter histogram).

Fixation

Fixation is very advantageous in a clinical setting where there may not be immediate access to a flow cytometer. Fixation prevents subsequent artefactual in vitro platelet activation. For most antibodies, the 'antibody labelling before fixation' method described above results in no significant differences in fluorescence intensity between samples analysed immediately and samples analysed within 24 hours of fixation[24]. A 'fixation before antibody labelling' method also results in no significant differences in fluorescence intensity between samples analysed immediately and samples analysed within 24 hours of antibody labelling[37]. However, fixation is an important variable to be controlled for, especially in a 'fixation before antibody labelling' method, because the binding of activation-dependent monoclonal antibodies to fixed platelets is often decreased compared to unfixed platelets[5]. Furthermore, the binding of some antibodies further decreases after fixation in a time-dependent manner. The optimal fixation method for each new monoclonal antibody must therefore be defined by each laboratory.

An argument in favour of immediate fixation is that activation-dependent changes are often time dependent, at least in vitro. For example, the platelet surface expression of the GPIb–IX–V complex decreases within 30 seconds of platelet activation, reaching a nadir at approximately 5 minutes, but over the next approximately 45 minutes the platelet surface expression of the GPIb–IX–V complex returns to normal[12,34]. The activation-dependent increase in the platelet surface expression of the GPIIb–IIIa complex and CD154 (CD40 ligand, CD40L) are also reversible with time[45,46]. In contrast, although circulating degranulated platelets rapidly lose their surface P-selectin in vivo[13], the activation-dependent increase in platelet surface P-selectin is not reversible over time in vitro[12,45].

Choice of monoclonal antibodies

Because the exposure of different antigens may reflect different aspects of platelet activation, it may be preferable to use a panel of monoclonal antibodies (see above). Monoclonal antibodies are preferable to polyclonal antibodies in whole blood flow cytometry, because they (i) can more reliably saturate all specific epitopes and (ii) result in less non-specific binding. Platelet-specific monoclonal antibodies are now available from a wide variety of commercial sources, and can often be purchased already conjugated to FITC, biotin, phycoerythrin, PerCP, APC, or a tandem conjugate, e.g. phycoerythrin-Cy5 or phycoerythrin-Texas Red. Alternatively, unlabelled antibodies can be FITC conjugated by the method of Rinderknecht[47] or (easier and more rapidly) by a kit method (commercially available from, for example, Boehringer Mannheim (Indianapolis, IN), Molecular Probes (Eugene, OR), and Sigma-Aldrich (St Louis, MO)). Antibodies can be biotinylated as described by Shattil et al.[24] or by following the biotin manufacturer's directions. The use of antibodies that are directly conjugated with FITC, phycoerythrin, PerCP, APC, or tandem conjugates eliminates the requirement for the addition of secondary antibodies, thereby avoiding time-consuming additional incubations and washing procedures which, in unfixed samples, may result in artefactual in vitro activation of platelets. Furthermore, the use of secondary antibodies is likely to result in increased background fluorescence and decreased sensitivity of the assay.

F_{ab} fragments of monoclonal antibodies can be used to avoid F_c-mediated binding and the F_c-induced platelet activation that has been reported with some monoclonal antibodies[48]. However, the use of F_{ab} fragments is usually unnecessary, provided: (i) the absence of antibody-induced binding of other activation-dependent monoclonal antibodies is demonstrated in control samples, or the problem is avoided by fixation before test antibody binding; (ii) the F_c-mediated and non-specific binding obtained from parallel samples with isotypic species-specific immunoglobulin is subtracted from the binding of the test antibody, or (iii) immunoglobulins, F_c fragments, or peptides are used as blocking reagents.

The saturating concentration of each antibody for platelet binding must be specifically determined by each laboratory. This concentration is typically between 0.25 and 20 μg/ml. In addition, when two monoclonal antibodies are used in the same assay (as is standard in whole blood flow cytometry), it is necessary to determine that they do not interfere with each other for platelet binding.

Platelets can be detected in whole blood by light scatter only. However, under certain experimental conditions, some of the particles falling within the light scatter gate for platelets may not bind any platelet-specific monoclonal antibody. It is therefore recommended that a minimum

Cambridge Books Online © Cambridge University Press, 2009

two colour/two antibody technique be used for whole blood flow cytometry: one monoclonal antibody (for example, GPIb-, GPIIb-, or GPIIIa-specific, typically phycoerythrin-conjugated) to identify a particle as a platelet; another monoclonal antibody (typically FITC conjugated) to quantify the expression of the glycoprotein of interest.

Expression of antibody binding

Antibody binding can be expressed as mean fluorescence intensity (MFI) or as the percent of platelets staining positive for a particular antibody (based on a positive analysis region placed just to the right of the negative control fluorescence histogram). Depending on the experimental circumstances and the physiologic nature of the antigen being measured, either MFI or percent positive platelets may have more relevance than the other. Unlike the MFI method, the 'per cent positive platelets' method is independent of variations in signal amplification, e.g. as a result of changes in PMT voltage or gain, because the isotypic control signal increases in proportion with the test sample. The 'per cent positive platelets' method may detect subpopulations of platelets arising from a local in vivo insult. However, it is important to realize that 'antibody-positive' platelets may have very little antigen expressed on their surface. For example, in a given clinical setting, the data may be reported as 20% circulating activated platelets, based on P-selectin positivity. However, if each P-selectin-positive platelet expresses only 10% of maximal platelet surface P-selectin, the overall average increase in platelet surface P-selectin is only 2%. If the goal is to determine the total amount of platelet surface antigen, MFI is therefore the preferred method of data presentation. For activation-dependent antibodies, inclusion of a control sample maximally activated by thrombin, TRAP, or phorbol myristate acetate assists in the quantification of the amount of surface antigen per platelet. The activation-dependent decrease in platelet surface GPIb–IX–V[25,34,49,50] should be quantified by MFI rather than by the 'percentage of positive platelets' method, because the decrease in platelet surface GPIb–IX–V on each platelet is usually insufficient to result in a 'negative' platelet. Methods have been described for the calculation of a binding index that takes into account both MFI and percent positive platelets[51].

Although standard flow cytometry does not result in a measure of the absolute number of binding sites, Shattil et al.[24] and Johnston et al.[52] used monoclonal antibodies double-labelled with ^{125}I and biotin to demonstrate a direct linear relationship between the number of antibody binding sites per platelet as determined by ^{125}I-labelled and (after incubation with phycoerythrin-streptavidin) fluorescent-labelled antibody. Once this relationship is known for a given monoclonal antibody, it is possible to use subsequent batches of the biotinylated or FITC-conjugated antibody for binding site quantitation, provided that the molar ratio of fluorescein to antibody is known.

Commercial kits, e.g. Quantum, Flow Cytometry Standards Corp., San Juan, PR, containing a set of calibrated fluorescent standards and software can be used to determine molecules of equivalent soluble fluorochrome (MESF). Use of these standards allows: (i) quantitation of the fluorescence intensity of samples in terms of MESF, (ii) determination of the fluorescence threshold of the instrument, (iii) determination of the linearity and stability of the instrument, and, most importantly, (iv) data comparison over time and between different instruments and laboratories[53]. Furthermore, flow cytometric methods are now available for the absolute quantitation of the number of antibodies bound per cell, e.g. Quantum Simply Cellular Microbeads Kit, Flow Cytometry Standards Corp. The lower limit of detection of antibody binding by flow cytometry is approximately 500 antibody molecules per platelet.

Calibration of the flow cytometer

To ensure day-to-day sample reproducibility, the flow cytometer should be calibrated daily using commercially available fluorescent beads. Daily confirmation of satisfactory instrument electronics, fluidics, and alignment should also be performed. Because of spectral emission overlap, the proper electronic color compensation must be set for each combination of antibodies (fluorophores) per the instructions of the manufacturer of the flow cytometer and confirmed by each laboratory.

Detection of the activation-dependent decrease in the platelet surface expression of the GPIb–IX–V complex

Whole blood flow cytometric assays frequently employ a GPIb-specific monoclonal antibody to identify platelets. Because GPIb is not present on any circulating blood cell except platelets[54,55], the activation-induced decrease in the platelet surface expression of GPIb[25,49,50] generally does not result in fluorescence below the threshold used to distinguish platelets from other cells and debris[24,28]. Thus, no subpopulations of platelets are excluded. A method of avoiding the activation-induced decrease in binding of a GPIb-specific monoclonal antibody is to add a direct conjugate of the GPIb-specific antibody before addition of the agonist[56] (see below).

Cambridge Books Online © Cambridge University Press, 2009

To specifically analyse the activation-induced decrease in the platelet surface expression of the GPIb–IX complex in whole blood, a GPIIb- or GPIIIa-specific monoclonal antibody can be employed as the platelet-identifying reagent[37].

There is an important methodologic point that needs to be emphasized with regard to the flow cytometric detection of the activation-induced decrease in the platelet surface expression of the GPIb–IX complex[56]. If a FITC-conjugated GPIb–IX-specific test monoclonal antibody is added prior to the platelet agonist (as in the typical schema shown in Table 32.1), the activation-induced redistribution of GPIb–IX to the surface-connected canalicular system[57] will not result in a significant decrease in platelet fluorescence, because a flow cytometer can detect FITC fluorescence, irrespective of whether the conjugated antibody is on the surface or the interior of the platelet. Therefore, in flow cytometric assays, GPIb–IX-specific antibodies that are directly conjugated, e.g. with FITC must be added to the assay after the addition of the agonist.

Clinical applications of whole blood flow cytometry

Platelet hyper-reactivity and/or circulating activated platelets in acute coronary syndromes

Whole blood flow cytometric studies have demonstrated circulating activated platelets in patients with unstable angina and acute myocardial infarction[58,59]. Whole blood flow cytometric studies have also demonstrated circulated activated platelets and increased platelet reactivity in patients with stable coronary artery disease[60]. In addition, coronary angioplasty results in platelet activation, as evidenced by whole blood flow cytometric analysis of coronary sinus blood[61,62].

Flow cytometric analysis of platelet activation-dependent markers can be used to determine optimal antiplatelet therapy in clinical settings, e.g. in acute coronary syndromes[63,64] and after coronary stenting[65,66]. Flow cytometric analysis of platelet activation markers before angioplasty may predict an increased risk of acute[67] and subacute[68] ischemic events after angioplasty.

Circulating activated platelets in cerebrovascular ischemia

Increased circulating P-selectin-positive and CD63-positive platelets have been reported in acute cerebrovascular ischemia[69]. Furthermore, increased expression of surface P-selectin on platelets is a risk factor for silent cerebral infarction in patients with atrial fibrillation[70].

Leukocyte–platelet aggregates

By a combination of platelet-specific and leukocyte-specific monoclonal antibodies, heterotypic aggregates, e.g. monocyte–platelet or neutrophil–platelet can be determined by flow cytometry[60,71]. Platelet degranulation with surface P-selectin exposure is necessary for the formation of these monocyte–platelet and neutrophil–platelet aggregates[72,73]. In the clinical settings of stable coronary artery disease, percutaneous coronary interventions, and venous insufficiency, circulating monocyte–platelet aggregates (but not neutrophil–platelet aggregates) are, in fact, a more sensitive marker of in vivo platelet activation than circulating P-selectin-positive platelets[60,74–76].

Circulating leukocyte–platelet aggregates are increased in acute myocardial infarction[77,78], unstable angina[79], stable coronary artery disease[60], and cardiopulmonary bypass[80]. Circulating leukocyte–platelet aggregates also increase after coronary angioplasty, with a greater magnitude in patients experiencing late clinical events[81].

Monitoring of GPIIb–IIIa antagonist therapy

GPIIb–IIIa antagonists are increasingly prescribed in clinical settings. Flow cytometric methods can be used to monitor this therapy by measuring receptor occupancy by these drugs. These methods can be categorized as either direct or indirect. One type of direct method is a competitive binding assay with either (i) biotinylated[82] or FITC-conjugated[83] GPIIb-IIIa antagonist (ii) blocking monoclonal antibodies[84,85] (iii) peptides with very high affinities for GPIIb–IIIa (disintegrins[86] or cyclic RGD peptides[87]); or, (iv) fibrinogen binding after platelet activation (detected by either a polyclonal anti-fibrinogen antibody[88] or by an anti-RIBS antibody [A.D. Michelson and M.R. Barnard, unpublished data]). Another type of direct method measures the binding of an antibody directed against the GPIIb–IIIa antagonist[89]. Indirect methods measure either (i) the GPIIb–IIIa antagonist-induced binding of an anti-LIBS antibody[90] or, (ii) platelet aggregation, as determined by light scatter[82].

Quality control of stored platelet concentrates in the blood bank

As determined by P-selectin, CD63, and the GPIIb–IIIa complex, flow cytometric studies of platelets stored in the blood bank prior to transfusion have provided direct

Cambridge Books Online © Cambridge University Press, 2009

evidence of a time-dependent platelet activation[91–93]. These changes correlated with modifications in platelet morphology (decrease in swirling), leakage of lactate dehydrogenase, and release of b-thromboglobulin[91]. It has been suggested that platelet surface P-selectin may be useful as a quality control measurement, based on correlations between the platelet surface expression of P-selectin in stored blood bank platelets and (i) post-transfusion platelet counts[94], (ii) platelet survival determined by In^{111} [92,95], and (iii) enhanced clearance of P-selectin-positive platelets from the recipient[92].

However, in vivo in a primate model, Michelson et al.[13] demonstrated that circulating degranulated platelets rapidly lose surface P-selectin but continue to circulate and function. This study was confirmed in a knockout mouse model by Berger et al.[14] who demonstrated that P-selectin, despite its binding to leukocytes, does not mediate platelet clearance in any of the following settings: physiological ageing, platelet activation, and platelet storage at 4 °C.

Platelet hyporeactivity

Although there are currently few published studies in this area, whole blood flow cytometry may be useful in the clinical assessment of platelet hyporeactivity. For example, compared to adults, the platelets of very low birthweight preterm neonates are markedly hyporeactive to thrombin, ADP/epinephrine and thromboxane A_2, as determined by flow cytometric detection of (i) the exposure of the fibrinogen binding site on the GPIIb–IIIa complex, (ii) fibrinogen binding, (iii) the increase in platelet surface P-selectin, and (iv) the decrease in platelet surface GPIb[29]. This platelet hyporeactivity may contribute to the propensity of very low birth weight neonates to intraventricular hemorrhage.

Diagnosis of inherited deficiencies of platelet surface glycoproteins

Flow cytometry provides a rapid and simple means for the diagnosis of the homozygous and heterozygous states of platelet membrane glycoprotein deficiencies, such as Bernard–Soulier syndrome (deficiency of GPIb–IX–V) and Glanzmann's thrombasthenia (deficiency of GPIIb–IIIa)[96,97]. Furthermore, in Bernard–Soulier syndrome, whole blood flow cytometry allows analysis of platelets without attempting the technically difficult procedure of physically separating the giant platelets from similarly-sized red and white blood cells. Because light scatter (especially forward light scatter) correlates with platelet size, light scatter gates may need to be adjusted in giant platelet syndromes, including Bernard–Soulier syndrome. This adjustment may result in overlap of the light scatter of giant platelets with red and white blood cells. It is therefore essential to include in the assay a platelet-specific monoclonal antibody as a platelet identifier. For Bernard–Soulier syndrome platelets, this identifier antibody obviously cannot be GPIb, GPIX, or GPV specific.

In addition, a panel of activation-dependent monoclonal antibodies can be used to evaluate patients with defects in platelet aggregation[98], secretion[99], or procoagulant activity[100].

Diagnosis of storage pool disease

Inherited dense granule storage pool deficiency, a relatively common cause of a mild hemorrhagic diathesis, cannot be reliably diagnosed by standard platelet aggregometry. However, using mepacrine-loaded platelets, storage pool deficiency can be accurately diagnosed by a simple, rapid, one-step flow cytometric assay in a clinical laboratory[101,102].

Reticulated platelets

Whole blood flow cytometric methods have been developed for the identification of young platelets, i.e. those containing mRNA by their staining with thiazole orange[103–105]. Because of the analogy to reticulocytes, these thiazole orange-positive platelets have been termed 'reticulated platelets'[104,106]. Thrombocytopenic patients whose bone marrow contained normal or increased numbers of megakaryocytes had significantly elevated proportions of circulating reticulated platelets[103]. In contrast, the proportion of reticulated platelets in thrombocytopenic patients with impaired platelet production (reduced bone marrow megakaryocytes) did not differ from normal controls and the absolute number of reticulated platelets was significantly lowered[103]. Measurement of reticulated platelets has been used as an aid in assessing bone marrow recovery after bone marrow transplantation[107]. Because thiazole orange also binds to ADP and ATP (contained in dense granules), important controls in the flow cytometric measurement of reticulated platelets are the demonstration that thiazole orange staining is (i) abolished by pretreatment of the sample with RNAase, and (ii) not abolished by pretreatment of the sample with thrombin.

Platelet-derived microparticles

As determined by flow cytometry, in vitro activation of platelets by some agonists, e.g. C5b-9, collagen/thrombin,

Cambridge Books Online © Cambridge University Press, 2009

and the calcium ionophore A23187 results in platelet-derived microparticles (defined by low forward angle light scatter and binding of a platelet-specific monoclonal antibody) that are procoagulant (determined by binding of monoclonal antibodies to activated factors V or VIII or by annexin V)[108,109]. These findings suggest that procoagulant platelet-derived microparticles may have an important role in the assembly of the 'tenase' and 'prothrombinase' components of the coagulation system in vivo.

Platelet-derived microparticles are present in blood bank fresh frozen plasma and cryoprecipitate[110]. Platelet-derived microparticles have been reported to be increased in the following clinical settings: immune thrombocytopenic purpura[111,112], transient ischemic attacks[113], acute coronary syndromes[114], and cardiopulmonary bypass[38,115]. Increased platelet microparticles and procoagulant activity occur in symptomatic patients with prosthetic heart valves and provided a potential pathophysiological explanation of cerebrovascular events in this patient group[116]. Procoagulant platelet-derived microparticles are decreased in preterm neonates compared with adults, and this may contribute to the propensity of preterm neonates to intraventricular hemorrhage[117]. GPIIb–IIIa antagonists also decrease procoagulant platelet-derived microparticles, and heparin and enoxaparin augment this reduction[118].

Diagnosis of heparin-induced thrombocytopenia

Unlike normal sera and sera from patients with quinine- or quinidine-induced thrombocytopenia, sera from patients with heparin-induced thrombocytopenia generate procoagulant platelet-derived microparticles from normal platelets[119]. This observation has been used to develop a rapid, specific, and sensitive flow cytometric assay for the diagnosis of heparin-induced thrombocytopenia[120].

Other potential clinical applications of flow cytometry

Other conditions in which whole blood flow cytometric measurement of platelet hyperreactivity and/or circulating activated platelets may prove to have a clinical role include pre-eclampsia[121,122], peripheral vascular disease[123], diabetes mellitus[124], and Alzheimer disease[125].

Because of the minuscule volumes of blood required, whole blood flow cytometry can be used to analyze the shed blood that emerges from a standardized bleeding time wound[25,37,38,126]. The time-dependent increase in the platelet surface expression of P-selectin in this shed blood reflects in vivo activation of platelets[25,37,38,126]. Immediate fixation (prior to antibody incubation) is obligatory, in order to observe these time-dependent changes. The assay can be used to demonstrate deficient platelet reactivity in response to an in vivo wound, for example during cardiopulmonary bypass[37].

Flow cytometry can also be used to: immunophenotype platelet HPA-1a[127]; detect maternal and fetal anti-HPA-1a antibodies[128]; measure platelet-associated IgG in immune thrombocytopenias[129] and alloimmunization[130]; and crossmatch platelets, which may be useful for alloimmunized patients for whom HLA compatible platelets are not readily available[131].

Flow cytometry can be used to measure platelet calcium flux with Indo-1[132,133] or Fluo-3/Fura Red ratios[134], including in whole blood[135,136]. Platelet cytoskeletal rearrangement can be analyzed flow cytometrically by measuring F-actin content with NBD- or bodipy-phallicidin or FITC-phalloidin[136,137].

Flow cytometry can be used to easily and precisely count very low numbers of platelets. A known number of fluorescently labelled microbeads is added to a given volume of diluted anticoagulated whole blood. After fluorescent labelling of the platelets, the platelet count can be calculated. This method is particularly advantageous for severely thrombocytopenic patients for whom standard Coulter counters are less accurate.

References

1 Michelson AD. Flow cytometry: a clinical test of platelet function. *Blood* 1996; 87: 4925–4936.
2 Michelson AD, Furman MI. Laboratory markers of platelet activation and their clinical significance. *Curr Opin Hematol* 1999; 6: 342–348.
3 Givan AL. *Flow cytometry. First principles.* New York: Wiley-Liss, 1992.
4 Schmitz G, Rothe G, Ruf A et al. European Working Group on Clinical Cell Analysis: consensus protocol for the flow cytometric characterisation of platelet function. *Thromb Haemost* 1998; 79: 885–896.
5 Michelson AD, Barnard MR, Benoit SE, Mitchell J, Knowles C, Ault KA. Characterization of platelet binding of blind panel mAb. In Schlossman SF, Boumsell L, Gilks W, Harlan JW, Kishimoto T, Morimoto C et al., eds. *Leucocyte typing* V. Oxford: Oxford University Press; 1995: 1207–1210.
6 Phillips DR, Charo IF, Parise LV, Fitzgerald LA. The platelet membrane glycoprotein IIb-IIIa complex. *Blood* 1988; 71: 831–843.
7 Shattil SJ, Hoxie JA, Cunningham M, Brass LF. Changes in the platelet membrane glycoprotein IIb-IIIa complex during platelet activation. *J Biol Chem* 1985; 260: 11107–11114.
8 Frelinger AL, Lam SC, Plow EF, Smith MA, Loftus JC, Ginsberg

Cambridge Books Online © Cambridge University Press, 2009

MH. Occupancy of an adhesive glycoprotein receptor modulates expression of an antigenic site involved in cell adhesion. *J Biol Chem* 1988; 263: 12397–12402.
9 Zamarron C, Ginsberg MH, Plow EF. Monoclonal antibodies specific for a conformationally altered state of fibrinogen. *Thromb Haemost* 1990; 64: 41–46.
10 Palabrica T, Lobb R, Furie BC et al. Leukocyte accumulation promoting fibrin deposition is mediated in vivo by P-selectin on adherent platelets. *Nature* 1992; 359: 848–851.
11 McEver RP. Properties of GMP-140, an inducible granule membrane protein of platelets and endothelium. *Blood Cells* 1990; 16: 73–80.
12 Michelson AD, Benoit SE, Kroll MH et al. The activation-induced decrease in the platelet surface expression of the glycoprotein Ib–IX complex is reversible. *Blood* 1994; 83: 3562–3573.
13 Michelson AD, Barnard MR, Hechtman HB et al. In vivo tracking of platelets: circulating degranulated platelets rapidly lose surface P-selectin but continue to circulate and function. *Proc Natl Acad Sci USA* 1996; 93: 11877–11882.
14 Berger G, Hartwell DW, Wagner DD. P-Selectin and platelet clearance. *Blood* 1998; 92: 4446–4452.
15 Kinlough-Rathbone RL, Packham MA, Mustard JF. Platelet aggregation. In Harker LA, Zimmerman TS, eds. *Measurements of platelet function*. New York: Churchill Livingstone; 1983: 64–91.
16 Kaplan KL, Owen J. Radioimmunoassays of platelet alpha-granule proteins. In Harker LA, Zimmerman TS, eds. *Measurements of platelet function*. Edinburgh: Churchill Livingstone; 1983: 115–125.
17 Levine SP. Secreted platelet proteins as markers for pathological disorders. In Phillips DR, Shuman MA, eds. *Biochemistry of platelets*. Orlando: Academic Press, 1986: 378–415.
18 Chong BH, Murray B, Berndt MC, Dunlop LC, Brighton T, Chesterman CN. Plasma P-selectin is increased in thrombotic consumptive platelet disorders. *Blood* 1994; 83: 1535–1541.
19 Fitzgerald DJ, Roy L, Catella F, FitzGerald GA. Platelet activation in unstable coronary disease. *N Engl J Med* 1986; 315: 983–989.
20 Oates JA, FitzGerald GA, Branch RA, Jackson EK, Knapp HR, Roberts LJ. Clinical implications of prostaglandin and thromboxane A2 formation. *N Engl J Med* 1988; 319: 689–698.
21 George JN, Shattil SJ. The clinical importance of acquired abnormalities of platelet function. *N Engl J Med* 1991; 324: 27–39.
22 Santos MT, Valles J, Marcus AJ et al. Enhancement of platelet reactivity and modulation of eicosanoid production by intact erythrocytes. A new approach to platelet activation and recruitment. *J Clin Invest* 1991; 87: 571–580.
23 LaRosa CA, Rohrer MJ, Rodino LJ, Benoit SE, Barnard MR, Michelson AD. Human neutrophil cathepsin G is a potent platelet activator. *J Vasc Surg* 1994; 19: 306–319.
24 Shattil SJ, Cunningham M, Hoxie JA. Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. *Blood* 1987; 70: 307–315.
25 Michelson AD, Ellis PA, Barnard MR, Matic GB, Viles AF, Kestin AS. Downregulation of the platelet surface glycoprotein Ib–IX complex in whole blood stimulated by thrombin, ADP or an in vivo wound. *Blood* 1991; 77: 770–779.
26 Abrams C, Shattil SJ. Immunological detection of activated platelets in clinical disorders. *Thromb Haemost* 1991; 65: 467–473.
27 Michelson AD. Platelet activation by thrombin can be directly measured in whole blood through the use of the peptide GPRP and flow cytometry: methods and clinical studies. *Blood Coagul Fibrinolysis* 1994; 5: 121–131.
28 Kestin AS, Ellis PA, Barnard MR, Errichetti A, Rosner BA, Michelson AD. The effect of strenuous exercise on platelet activation state and reactivity. *Circulation* 1993; 88: 1502–1511.
29 Rajasekhar D, Barnard MR, Bednarek FJ, Michelson AD. Platelet hyporeactivity in very low birth weight neonates. *Thromb Haemost* 1997; 77: 1002–1007.
30 Hansen SR, Harker LA. Interruption of acute platelet-dependent thrombosis by the synthetic antithrombin PPACK. *Proc Natl Acad Sci USA* 1988; 85: 3184–3188.
31 Eidt JF, Allison P, Nobel S et al. Thrombin is an important mediator of platelet aggregation in stenosed canine coronary arteries with endothelial injury. *J Clin Invest* 1989; 84: 18–27.
32 Kelly AB, Marzec UM, Krupski W et al. Hirudin interruption of heparin-resistant arterial thrombus formation in baboons. *Blood* 1991; 77: 1006–1012.
33 Vu T-KH, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* 1991; 64: 1057–1068.
34 Michelson AD, Benoit SE, Furman MI, Barnard MR, Nurden P, Nurden AT. The platelet surface expression of glycoprotein V is regulated by two independent mechanisms: proteolysis and a reversible cytoskeletal-mediated redistribution to the surface-connected canalicular system. *Blood* 1996; 87: 1396–1408.
35 Yamamoto N, Greco NJ, Barnard MR et al. Glycoprotein Ib (GPIb)-dependent and GPIb-independent pathways of thrombin-induced platelet activation. *Blood* 1991; 77: 1740–1748.
36 Kahn ML, Zheng YW, Huang W et al. A dual thrombin receptor system for platelet activation. *Nature* 1998; 394: 690–694.
37 Kestin AS, Valeri CR, Khuri SF et al. The platelet function defect of cardiopulmonary bypass. *Blood* 1993; 82: 107–117.
38 Abrams CS, Ellison N, Budzynski AZ, Shattil SJ. Direct detection of activated platelets and platelet-derived microparticles in humans. *Blood* 1990; 75: 128–138.
39 Mammen EF, Koets MH, Washington BC et al. Hemostasis changes during cardiopulmonary bypass surgery. *Semin Thromb Haemost* 1985; 11: 281–292.
40 Harker LA, Malpass TW, Branson HE, Hessel EA, Slichter SJ. Mechanism of abnormal bleeding in patients undergoing

Cambridge Books Online © Cambridge University Press, 2009

cardiopulmonary bypass: acquired transient platelet dysfunction associated with selective a-granule release. *Blood* 1980; 56: 824–834.
41 Ault KA, Rinder HM, Mitchell JG, Rinder CS, Lambrew CT, Hillman RS. Correlated measurement of platelet release and aggregation in whole blood. *Cytometry* 1989; 10: 448–455.
42 Evangelista V, Manarini S et al. Platelet/polymorphonuclear leukocyte interaction in dynamic conditions: evidence of adhesion cascade and cross talk between P-selectin and the beta 2 integrin CD11b/CD18. *Blood* 1996; 88: 4183–4194.
43 Ciferri S, Emiliani C, Guglielmini G, Orlacchio A, Nenci GG, Gresele P. Platelets release their lysosomal content in vivo in humans upon activation. *Thromb Haemost* 2000; 83: 157–164.
44 Frelinger AL, Cohen I, Plow EF et al. Selective inhibition of integrin function by antibodies specific for ligand-occupied receptor conformers. *J Biol Chem* 1990; 265: 6346–6352.
45 Ruf A, Patscheke H. Flow cytometric detection of activated platelets: comparison of determining shape change, fibrinogen binding, and P-selectin expression. *Semin Thromb Haemost* 1995; 21: 146–151.
46 Henn V, Slupsky JR, Grafe M et al. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 1998; 391: 591–594.
47 Rinderknecht H. Ultra-rapid fluorescent labelling of proteins. *Nature* 1962; 193: 167–168.
48 Horsewood P, Hayward CP, Warkentin TE, Kelton JG. Investigation of the mechanisms of monoclonal antibody-induced platelet activation. *Blood* 1991; 78: 1019–1026.
49 Michelson AD. Thrombin-induced down-regulation of the platelet membrane glycoprotein Ib–IX complex. *Semin Thromb Haemost* 1992; 18: 18–27.
50 Michelson AD, Barnard MR. Thrombin-induced changes in platelet membrane glycoproteins Ib, IX, and IIb–IIIa complex. *Blood* 1987; 70: 1673–1678.
51 Leytin V, Mody M, Semple JW, Garvey B, Freedman J. Flow cytometric parameters for characterizing platelet activation by measuring P-selectin (CD62) expression: theoretical consideration and evaluation in thrombin-treated platelet populations. *Biochem Biophys Res Commun* 2000; 269: 85–90.
52 Johnston GI, Pickett EB, McEver RP, George JN. Heterogeneity of platelet secretion in response to thrombin demonstrated by fluorescence flow cytometry. *Blood* 1987; 69: 1401–1403.
53 Poon RYM, Johnson R, Stanton T, Fernandez-Repollet E, Schwartz A. Calibration of fluorescent intensity microbead standards and assignment of effective fluorescein/protein ratios to the fluorescein isothiocyanate (FITC)-conjugated secondary antibodies used in the Fifth Workshop. In Schlossman SF, Boumsell L, Gilks W, Harlan JM, Kishimoto T, Morimoto C et al., eds. *Leucocyte typing* V. Oxford: Oxford University Press; 1995: 13–16.
54 Coller BS, Peerschke EI, Scudder LE, Sullivan CA. Studies with a murine monoclonal antibody that abolishes ristocetin-induced binding of von Willebrand factor to platelets: additional evidence in support of GPIb as a platelet receptor for von Willebrand factor. *Blood* 1983; 61: 99–110.
55 Montgomery RR, Kunicki TJ, Taves C, Pidard D, Corcoran M. Diagnosis of Bernard–Soulier syndrome and Glanzmann's thrombasthenia with a monoclonal assay on whole blood. *J Clin Invest* 1983; 71: 385–389.
56 Michelson AD, Barnard MR. Flow cytometric detection of the redistribution of the glycoprotein Ib–IX complex on thrombin-stimulated platelets is dependent on the type of antibody conjugate used. *Blood* 1993; 81: 1408–1409.
57 Hourdille P, Heilmann E, Combrie R, Winckler J, Clemetson KJ, Nurden AT. Thrombin induces a rapid redistribution of glycoprotein Ib–IX complexes within the membrane systems of activated human platelets. *Blood* 1990; 76: 1503–1513.
58 Becker RC, Tracy RP, Bovill EG, Mann KG, Ault K. The clinical use of flow cytometry for assessing platelet activation in acute coronary syndromes. TIMI-III Thrombosis and Anticoagulation Group. *Coron Artery Dis* 1994; 5: 339–345.
59 Schultheiss HP, Tschoepe D, Esser J et al. Large platelets continue to circulate in an activated state after myocardial infarction. *Eur J Clin Invest* 1994; 24: 243–247.
60 Furman MI, Benoit SE, Barnard MR et al. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. *J Am Coll Cardiol* 1998; 31: 352–358.
61 Scharf RE, Tomer A, Marzec UM, Teirstein PS, Ruggeri ZM, Harker LA. Activation of platelets in blood perfusing angioplasty-damaged coronary arteries. Flow cytometric detection. *Arterioscler Thromb* 1992; 12: 1475–1487.
62 Langford EJ, Brown AS, Wainwright RJ et al. Inhibition of platelet activity by S-nitrosoglutathione during coronary angioplasty. *Lancet* 1994; 344: 1458–1460.
63 Langford EJ, Wainwright RJ, Martin JF. Platelet activation in acute myocardial infarction and unstable angina is inhibited by nitric oxide donors. *Arterioscler Thromb Vasc Biol* 1996; 16: 51–55.
64 Ault KA, Cannon CP, Mitchell J et al. Platelet activation in patients after an acute coronary syndrome: results from the TIMI-12 trial. Thrombolysis in Myocardial Infarction. *J Am Coll Cardiol* 1999; 33: 634–639.
65 Gawaz M, Neumann FJ, Ott I, May A, Schomig A. Platelet activation and coronary stent implantation. Effect of antithrombotic therapy. *Circulation* 1996; 94: 279–285.
66 Neumann FJ, Gawaz M, Dickfeld T et al. Antiplatelet effect of ticlopidine after coronary stenting. *J Am Coll Cardiol* 1997; 29: 1515–1519.
67 Tschoepe D, Schultheib HP, Kolarov P et al. Platelet membrane activation markers are predictive for increased risk of acute ischemic events after PTCA. *Circulation* 1993; 88: 37–42.
68 Gawaz M, Neumann FJ, Ott I, May A, Rudiger S, Schomig A. Role of activation-dependent platelet membrane glycoproteins in development of subacute occlusive coronary stent thrombosis. *Coron Artery Dis* 1997; 8: 121–128.
69 Grau AJ, Ruf A, Vogt A et al. Increased fraction of circulating

Cambridge Books Online © Cambridge University Press, 2009

activated platelets in acute and previous cerebrovascular ischemia. *Thromb Haemost* 1998; 80: 298–301.

70 Minamino T, Kitakaze M, Sanada S et al. Increased expression of P-selectin on platelets is a risk factor for silent cerebral infarction in patients with atrial fibrillation: role of nitric oxide. *Circulation* 1998; 98: 1721–1727.

71 Li N, Goodall AH, Hjemdahl P. A sensitive flow cytometric assay for circulating platelet- leucocyte aggregates. *Br J Haematol* 1997; 99: 808–816.

72 Larsen E, Celi A, Gilbert GE et al. PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. *Cell* 1989; 59: 305–312.

73 Hamburger SA, McEver RP. GMP-140 mediates adhesion of stimulated platelets to neutrophils. *Blood* 1990; 75: 550–554.

74 Powell CC, Rohrer MJ, Barnard MR, Peyton BD, Furman MI, Michelson AD. Chronic venous insufficiency is associated with increased platelet and monocyte activation and aggregation. *J Vasc Surg* 1999; 30: 844–851.

75 Furman MI, Krueger LA, Barnard MR, Michelson AD. Circulating monocyte-platelet aggregates are a more sensitive marker of platelet activation than P-selectin-positive platelets. *Circulation* 2000; 102: II–777.

76 Peyton BD, Rohrer MJ, Furman MI et al. Patients with venous stasis ulceration have increased monocyte-platelet aggregation. *J Vasc Surg* 1998; 27: 1109–1116.

77 Gawaz M, Reininger A, Neumann FJ. Platelet function and platelet-leukocyte adhesion in symptomatic coronary heart disease. Effects of intravenous magnesium. *Thromb Res* 1996; 83: 341–349.

78 Neumann FJ, Marx N, Gawaz M et al. Induction of cytokine expression in leukocytes by binding of thrombin-stimulated platelets. *Circulation* 1997; 95: 2387–2394.

79 Ott I, Neumann FJ, Gawaz M, Schmitt M, Schomig A. Increased neutrophil-platelet adhesion in patients with unstable angina. *Circulation* 1996; 94: 1239–1246.

80 Rinder CS, Bonan JL, Rinder HM, Mathew J, Hines R, Smith BR. Cardiopulmonary bypass induces leukocyte-platelet adhesion. *Blood* 1992; 79: 1201–1205.

81 Mickelson JK, Lakkis NM, Villarreal-Levy G, Hughes BJ, Smith CW. Leukocyte activation with platelet adhesion after coronary angioplasty: a mechanism for recurrent disease? *J Am Coll Cardiol* 1996; 28: 345–353.

82 Konstantopoulos K, Kamat SG, Schafer AI et al. Shear-induced platelet aggregation is inhibited by in vivo infusion of an anti-glycoprotein IIb/IIIa antibody fragment, c7E3 Fab, in patients undergoing coronary angioplasty. *Circulation* 1995; 91: 1427–1431.

83 Gawaz M, Ruf A, Neumann F-J et al. Effect of glycoprotein IIb-IIIa receptor antagonism on platelet membrane glycoproteins after coronary stent placement. *Thromb Haemost* 1998; 80: 994–1001.

84 Quinn M, Deering A, Stewart M, Cox D, Foley B, Fitzgerald D. Quantifying GPIIb/IIIa receptor binding using 2 monoclonal antibodies: discriminating abciximab and small molecular weight antagonists. *Circulation* 1999; 99: 2231–2238.

85 Hezard N, Metz D, Nazeyrollas P et al. Free and total platelet glycoprotein IIb/IIIa measurement in whole blood by quantitative flow cytometry during and after infusion of c7E3 Fab in patients undergoing PTCA. *Thromb Haemost* 1999; 81: 869–873.

86 Liu CZ, Hur BT, Huang TF. Measurement of glycoprotein IIb/IIIa blockade by flow cytometry with fluorescein isothiocyanate-conjugated crotavirin, a member of disintegrins. *Thromb Haemost* 1996; 76: 585–591.

87 Tsao PW, Bozarth JM, Jackson SA, Forsythe MS, Flint SK, Mousa SA. Platelet GPIIb/IIIa receptor occupancy studies using a novel fluoresceinated cyclic Arg–Gly–Asp peptide. *Thromb Res* 1995; 77: 543–556.

88 Wittig K, Rothe G, Schmitz G. Inhibition of fibrinogen binding and surface recruitment of GpIIb/IIIa as dose-dependent effects of the RGD-mimetic MK-852. *Thromb Haemost* 1998; 79: 625–630.

89 Mascelli MA, Lance ET, Damaraju L et al. Pharmacodynamic profile of short-term abciximab treatment demonstrates prolonged platelet inhibition with gradual recovery from GP IIb/IIIa receptor blockade. *Circulation* 1998; 97: 1680–1688.

90 Jennings LK, White MM. Expression of ligand-induced binding sites on glycoprotein IIb/IIIa complexes and the effect of various inhibitors. *Am Heart J* 1998; 135: S179–S183.

91 Fijnheer R, Modderman PW, Veldman H et al. Detection of platelet activation with monoclonal antibodies and flow cytometry. Changes during platelet storage. *Transfusion* 1990; 30: 20–25.

92 Rinder HM, Murphy M, Mitchell JG, Stocks J, Ault KA, Hillman RS. Progressive platelet activation with storage: evidence for shortened survival of activated platelets after transfusion. *Transfusion* 1991; 31: 409–414.

93 Divers SG, Kannan K, Stewart RM et al. Quantitation of CD62, soluble CD62, and lysosome-associated membrane proteins 1 and 2 for evaluation of the quality of stored platelet concentrates. *Transfusion* 1995; 35: 292–297.

94 Triulzi DJ, Kickler TS, Braine HG. Detection and significance of alpha granule membrane protein 140 expression on platelets collected by apheresis. *Transfusion* 1992; 32: 529–533.

95 Ault KA, Mitchell J, Hillman RS. Appearance of P-selectin (CD62) during labeling is responsible for the majority of the variability in recovery of In111 labeled platelets. *Blood* 1992; 80: 496a.

96 Michelson AD. Flow cytometric analysis of platelet surface glycoproteins: Phenotypically distinct subpopulations of platelets in children with chronic myeloid leukemia. *J Lab Clin Med* 1987; 110: 346–354.

97 Jennings LK, Ashmun RA, Wang WC, Dockter ME. Analysis of human platelet glycoproteins IIb–IIIa and Glanzmann's thrombasthenia in whole blood by flow cytometry. *Blood* 1986; 68: 173–179.

98 Ginsberg MH, Frelinger AL, Lam SC et al. Analysis of platelet aggregation disorders based on flow cytometric analysis of membrane glycoprotein IIb-IIIa with conformation-specific monoclonal antibodies. *Blood* 1990; 76: 2017–2023.

Cambridge Books Online © Cambridge University Press, 2009

99 Lages B, Shattil SJ, Bainton DF, Weiss HJ. Decreased content and surface expression of alpha-granule membrane protein GMP-140 in one of two types of platelet alpha delta storage pool deficiency. *J Clin Invest* 1991; 87: 919–929.
100 Sims PJ, Wiedmer T, Esmon CT, Weiss HJ, Shattil SJ. Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome: an isolated defect in platelet procoagulant activity. *J Biol Chem* 1989; 264: 17049–17057.
101 Gordon N, Thom J, Cole C, Baker R. Rapid detection of hereditary and acquired platelet storage pool deficiency by flow cytometry. *Br J Haematol* 1995; 89: 117–123.
102 Wall JE, Buijs-Wilts M, Arnold JT et al. A flow cytometric assay using mepacrine for study of uptake and release of platelet dense granule contents. *Br J Haematol* 1995; 89: 380–385.
103 Kienast J, Schmitz G. Flow cytometric analysis of thiazole orange uptake by platelets: a diagnostic aid in the evaluation of thrombocytopenic disorders. *Blood* 1990; 75: 116–121.
104 Bonan JL, Rinder HM, Smith BR. Determination of the percentage of thiazole orange (TO)-positive, reticulated platelets using autologous erythrocyte TO fluorescence as an internal standard. *Cytometry* 1993; 14: 690–694.
105 Matic GB, Chapman ES, Zaiss M, Rothe G, Schmitz G. Whole blood analysis of reticulated platelets: improvements of detection and assay stability. *Cytometry* 1998; 34: 229–234.
106 Ault KA, Rinder HM, Mitchell J, Carmody MB, Vary CP, Hillman RS. The significance of platelets with increased RNA content (reticulated platelets). A measure of the rate of thrombopoiesis. *Am J Clin Pathol* 1992; 98: 637–646.
107 Romp KG, Peters WP, Hoffman M. Reticulated platelet counts in patients undergoing autologous bone marrow transplantation: an aid in assessing marrow recovery. *Am J Hematol* 1994; 46: 319–324.
108 Sims PJ, Faioni EM, Wiedmer T, Shattil SJ. Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity. *J Biol Chem* 1988; 263: 18205–18212.
109 Gilbert GE, Sims PJ, Wiedmer T, Furie B, Furie BC, Shattil SJ. Platelet-derived microparticles express high affinity receptors for factor VIII. *J Biol Chem* 1991; 266: 17261–17268.
110 George JN, Pickett EB, Heinz R. Platelet membrane microparticles in blood bank fresh frozen plasma and cryoprecipitate. *Blood* 1986; 68: 307–309.
111 Zucker-Franklin D, Karpatkin S. Red-cell and platelet fragmentation in idiopathic autoimmune thrombocytopenic purpura. *N Engl J Med* 1977; 297: 517–523.
112 Jy W, Horstman LL, Arce M, Ahn YS. Clinical significance of platelet microparticles in autoimmune thrombocytopenias. *J Lab Clin Med* 1992; 119: 334–345.
113 Lee YJ, Jy W, Horstman LL et al. Elevated platelet microparticles in transient ischemic attacks, lacunar infarcts, and multi-infarct dementias. *Thromb Res* 1994; 72: 295–304.
114 Katopodis JN, Kolodny L, Jy W, Horstman LL et al. Platelet microparticles and calcium homeostasis in acute coronary ischemias. *Am J Hematol* 1997; 54: 95–101.
115 George JN, Pickett EB, Saucerman S et al. Platelet surface glycoproteins. Studies on resting and activated platelets and platelet membrane microparticles in normal subjects, and observations in patients during adult respiratory distress syndrome and cardiac surgery. *J Clin Invest* 1986; 78: 340–348.
116 Geiser T, Sturzenegger M, Genewein U, Haeberli A, Beer JH. Mechanisms of cerebrovascular events as assessed by procoagulant activity, cerebral microemboli, and platelet microparticles in patients with prosthetic heart valves. *Stroke* 1998; 29: 1770–1777.
117 Michelson AD, Rajasekhar D, Bednarek FJ, Barnard MR. Platelet and platelet-derived microparticle surface factor V/Va binding in whole blood: differences between neonates and adults. *Thromb Haemost* 2000; 84: 689–694.
118 Furman MI, Krueger LA, Frelinger AL, III et al. GPIIb-IIIa antagonist-induced reduction in platelet surface factor V/Va binding and phosphatidylserine expression in whole blood. *Thromb Haemost* 2000; 84: 492–498.
119 Warkentin TE, Hayward CP, Boshkov LK et al. Sera from patients with heparin-induced thrombocytopenia generate platelet-derived microparticles with procoagulant activity: an explanation for the thrombotic complications of heparin-induced thrombocytopenia. *Blood* 1994; 84: 3691–3699.
120 Tomer A. A sensitive and specific functional flow cytometric assay for the diagnosis of heparin-induced thrombocytopenia. *Br J Haematol* 1997; 98: 648–656.
121 Janes SL, Goodall AH. Flow cytometric detection of circulating activated platelets and platelet hyper-responsiveness in pre-eclampsia and pregnancy. *Clin Sci* 1994; 86: 731–739.
122 Konijnenberg A, van der Post JA, Mol BW et al. Can flow cytometric detection of platelet activation early in pregnancy predict the occurrence of preeclampsia? A prospective study. *Am J Obstet Gynecol* 1997; 177: 434–442.
123 Devine DV, Andestad G, Nugent D, Carter CJ. Platelet-associated factor XIII as a marker of platelet activation in patients with peripheral vascular disease. *Arterioscler Thromb* 1993; 13: 857–862.
124 Tschoepe D, Roesen P, Esser J et al. Large platelets circulate in an activated state in diabetes mellitus. *Semin Thromb Haemost* 1991; 17: 433–438.
125 Sevush S, Jy W, Horstman LL, Mao WW, Kolodny L, Ahn YS. Platelet activation in Alzheimer disease. *Arch Neurol* 1998; 55: 530–536.
126 Michelson AD, MacGregor H, Barnard MR, Kestin AS, Rohrer MJ, Valeri CR. Reversible inhibition of human platelet activation by hypothermia in vivo and in vitro. *Thromb Haemost* 1994; 71: 633–640.
127 Forsberg B, Jacobsson S, Stockelberg D, Kutti J, Rydberg L, Wadenvik H. The platelet-specific alloantigen PlA1 (HPA-1a): a comparison of flow cytometric immunophenotyping and genotyping using polymerase chain reaction and restriction fragment length polymorphism in a Swedish blood donor population. *Transfusion* 1995; 35: 241–246.

Cambridge Books Online © Cambridge University Press, 2009

128 Marshall LR, Brogden FE, Roper TS, Barr AL. Antenatal platelet antibody testing by flow cytometry – results of a pilot study. *Transfusion* 1994; 34: 961–965.

129 Holme S, Heaton A, Kunchuba A, Hartman P. Increased levels of platelet associated IgG in patients with thrombocytopenia are not confined to any particular size class of platelets. *Br J Haematol* 1988; 68: 431–436.

130 Rosenfeld CS, Bodensteiner DC. Detection of platelet alloantibodies by flow cytometry. Characterization and clinical significance. *Am J Clin Pathol* 1986; 85: 207–212.

131 Gates K, MacPherson BR. Retrospective evaluation of flow cytometry as a platelet crossmatching procedure. *Cytometry* 1994; 18: 123–128.

132 Davies TA, Drotts D, Weil GJ, Simons ER. Flow cytometric measurements of cytoplasmic calcium changes in human platelets. *Cytometry* 1988; 9: 138–142.

133 Jennings LK, Dockter ME, Wall CD, Fox CF, Kennedy DM. Calcium mobilization in human platelets using indo-1 and flow cytometry. *Blood* 1989; 74: 2674–2680.

134 Novak EJ, Rabinovitch PS. Improved sensitivity in flow cytometric intracellular ionized calcium measurement using fluo-3/Fura Red fluorescence ratios. *Cytometry* 1994; 17: 135–141.

135 do Ceu MM, Sansonetty F, Goncalves MJ, O'Connor, JE. Flow cytometric kinetic assay of calcium mobilization in whole blood platelets using Fluo-3 and CD41. *Cytometry* 1999; 35: 302–310.

136 Cattaneo M, Kinlough-Rathbone RL, Lecchi A, Bevilacqua C, Packham MA, Mustard JF. Fibrinogen-independent aggregation and deaggregation of human platelets: studies of two afibrinogenemic patients. *Blood* 1993; 70: 221–223.

137 LaRosa CA, Rohrer MJ, Benoit SE, Barnard MR, Michelson AD. Neutrophil cathepsin G modulates the platelet surface expression of the glycoprotein (GP) Ib–IX complex by proteolysis of the von Willebrand factor binding site on GPIba and by a cytoskeletal-mediated redistribution of the remainder of the complex. *Blood* 1994; 84: 158–168.

138 Gralnick HR, Williams SB, McKeown L et al. Endogenous platelet fibrinogen: its modulation after surface expression is related to size-selective access to and conformational changes in the bound fibrinogen. *Br J Haematol* 1992; 80: 347–357.

139 Stenberg PE, McEver RP, Shuman MA, Jacques YV, Bainton DF. A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. *J Cell Biol* 1985; 101: 880–886.

140 Nieuwenhuis HK, van Oosterhout JJ, Rozemuller E, van Iwaarden F, Sixma JJ. Studies with a monoclonal antibody against activated platelets: evidence that a secreted 53 000-molecular weight lysosome-like granule protein is exposed on the surface of activated platelets in the circulation. *Blood* 1987; 70: 838–845.

141 Febbraio M, Silverstein RL. Identification and characterization of LAMP-1 as an activation-dependent platelet surface glycoprotein. *J Biol Chem* 1990; 265: 18531–18537.

142 Boukerche H, McGregor JL. Characterization of an anti-thrombospondin monoclonal antibody (P8) that inhibits human blood platelet functions. Normal binding of P8 to thrombin-activated Glanzmann thrombasthenic platelets. *Eur J Biochem* 1988; 171: 383–392.

143 Aiken ML, Ginsberg MH, Plow EF. Mechanisms for expression of thrombospondin on the platelet cell surface. *Semin Thromb Haemost* 1987; 13: 307–316.

144 Hayward CP, Smith JW, Horsewood P, Warkentin TE, Kelton JG. p-155, a multimeric platelet protein that is expressed on activated platelets. *J Biol Chem* 1991; 266: 7114–7120.

145 Hayward CP, Bainton DF, Smith JW et al. Multimerin is found in the alpha-granules of resting platelets and is synthesized by a megakaryocytic cell line. *J Clin Invest* 1993; 91: 2630–2639.

146 Michelson AD, Shattil SJ. The use of flow cytometry to study platelet activation. In Watson SP, Authi K, eds. *Platelet function and signal transduction: a practical approach.* Oxford: Oxford University Press; 1996: 111–129.

Cambridge Books Online © Cambridge University Press, 2009

33

Animal models of platelet-dependent thrombosis

Paolo Golino and Francesco Loffredo

Department of Internal Medicine, Division of Cardiology, University of Naples Federico II, Naples, Italy.

Introduction

Acute thrombotic occlusion of an atherosclerotic coronary or cerebral artery, leading to myocardial infarction or stroke, respectively, is still the leading cause of mortality in the Western world. Furthermore, acute thrombotic complications seriously compromise modern interventional approaches to the management of atherosclerotic vascular disease. Therefore, the development of effective antithrombotic therapies for acute arterial thrombotic occlusion still represents an important public health strategy.

Our understanding of the basic molecular processes involved in the formation and dissolution of thrombus has expanded rapidly in recent years, and this basic research has led to the generation of novel, potent, and clinically relevant antithrombotic agents, such as tissue plasminogen activator and its mutant forms, ticlopidine and clopidogrel, and Gp IIb/IIIa inhibitors. These agents are currently part of the clinical armamentarium available to the clinical cardiologist for the treatment of thrombotic disorders. With no doubts, therefore, the evaluation of these – as well as newer and perhaps even more effective – molecules requires relevant quantitative animal models of acute thrombosis; the ultimate application of new antithrombotic pharmacological agents being developed through the use of modern technology depends on the appropriate assessment in vivo to ensure their efficacy and safety[1].

Thus far, however, it has not been possible to reproduce, in an experimental model, the most common clinical cause of arterial thrombosis – an ulcerated or fissurated atherosclerotic plaque. Although during the past few years many animal models have been developed, the ideal model is far from being available, and this limitation should always be kept in mind. On the other hand, however, it should be also clear that these models represent an invaluable tool for the study of the pathophysiological mechanisms involved in the process of thrombus formation, as well as for the preclinical screening of new antithrombotic compounds; without the aid of these animal models it would not have been possible to develop antithrombotic compounds, such as tissue plasminogen activator or abciximab, which represent a cornerstone of the modern antithrombotic therapy. The purpose of this chapter is to describe the most commonly used animal models of intravascular thrombus formation and thrombolysis, pointing out the salient features of each model, including their advantages and drawbacks.

The Folts' recurrent coronary thrombosis model

In 1976 Folts and coworkers[2] described a model of repetitive thrombus formation in stenosed coronary arteries of open chest, anesthetized dogs. Subsequent to that description, other groups of investigators have used the Folts model, not only to test the antithrombotic effects of a variety of drugs, but also to investigate the mechanisms responsible for the interaction between platelets/coagulation factors and the vascular wall, ultimately leading to thrombus formation; the model is now extensively employed by investigators in the field of intravascular thrombosis in a variety of arteries, as well as in different species.

After opening of the chest at the fifth intercostal space under general anesthesia, the heart is suspended in a pericardial cradle. Either the left anterior descending (LAD) or the circumflex (Cx) coronary artery is isolated from the surrounding tissue. Next, endothelial and medial damage is produced by squeezing the artery between a pair of rubber-covered forceps and a plastic constrictor is placed around the injured arterial segment in order to obtain a

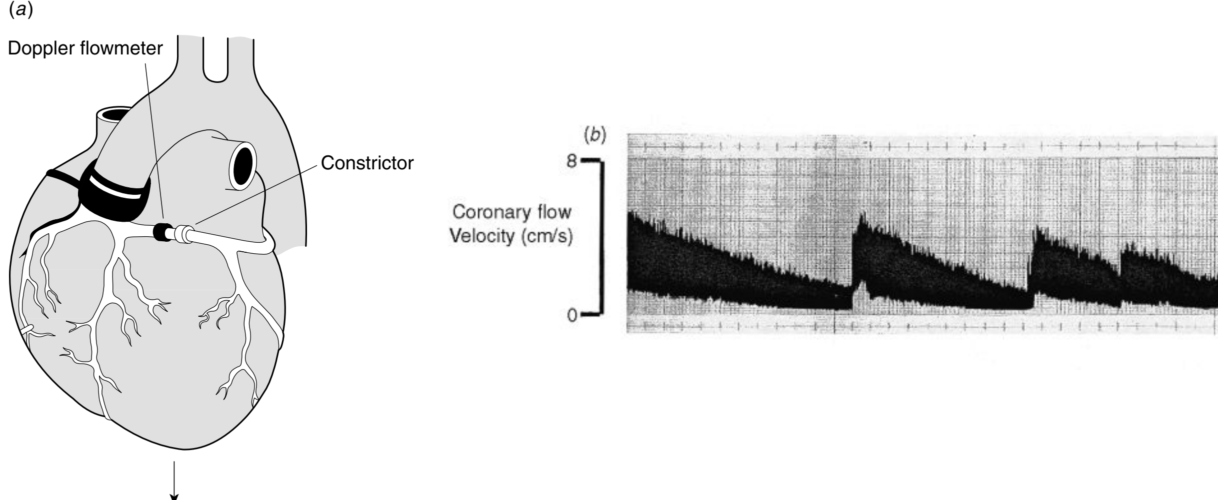


Fig. 33.1. Schematic representation of the experimental preparation of recurrent intravascular thrombosis. This model is thought to mimic the clinical situation of unstable angina. (*a*) Schematic representation of surgical preparation of the dog coronary artery. A short segment of coronary artery is exposed surgically under general anesthesia. Endothelial injury is produced and a plastic constrictor is then placed around the de-endothelialized segment to create a stenosis of about the 75% of the luminal diameter. Coronary blood flow velocity is measured continuously by a Doppler flow probe placed proximal to the constrictor. (*b*) In this model, cyclic flow variations (CFVs), characterized by gradual decrease in blood flow to almost zero values followed by restoration of flow to normal values usually develop within few minutes after placement of the constrictor.

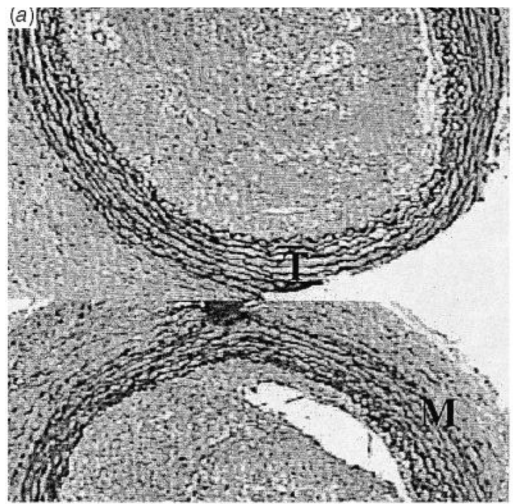
stenosis of about 70% of the luminal diameter. An electromagnetic or ultrasonic Doppler flowmeter probe of appropriate size is placed around the proximal coronary artery for measuring volume flow or flow velocity (Fig 33.1). Within a few minutes after instrumentation, the large majority of the animals develop a typical pattern of flow characterized by gradual decreases in blood flow to almost zero values, followed by sudden restorations of flow to baseline values (Fig. 33.1). This pattern of flow has been defined in terms of cyclic flow reductions (CFRs).

In this model, recurrent intravascular thrombosis originates from the combination of two thrombogenic stimuli: a hemodynamically significant stenosis and focal, intimal and medial injury. With very few exceptions, CFRs will not develop unless both stimuli are provided. An interesting feature of this model is that it closely mimics the pathophysiological conditions encountered in patients with acute coronary syndromes, i.e. unstable angina and acute myocardial infarction, in whom a thrombogenic surface is exposed to flowing blood under conditions of high shear stress, like those found in close proximity of a complicated atherosclerotic plaque[3,4].

After damaging and stenosing the coronary artery sufficiently, coronary blood flow starts declining immediately, reaching zero within a few minutes and remaining there until there is a deliberate, manual restoration of flow. This is usually accomplished by either flicking the constrictor or sliding the constrictor up and down the artery to mechanically dislodge the thrombus (Fig. 33.1). Spontaneous flow restorations may also occur, but usually under non-severe conditions, i.e. minimal stenosis or damage, or after administration of a partially effective antithrombotic agent.

Several groups have examined histologically the coronary arteries harvested from dogs undergoing CFRs: extensive intimal and medial injury, including de-endothelialization with rupture of the internal elastic lamina and various degree of medial tears with adherent platelets and/or microthrombi, are consistently observed (Fig. 33.2). Arteries harvested when coronary flow is at its nadir invariably reveal a platelet-rich thrombus filling the stenotic segment (Fig. 33.2)[2,5–11]. These histologic observations, coupled with the pattern of gradual, progressive declines in coronary flow and abrupt increases thereof (whether spontaneous or induced), provide further evidence that CFRs are indeed caused by progressive accumulation of platelet thrombi. Folts et al.[6] have provided angiographic evidence that CFRs are thrombotic by demonstrating a temporal relationship between cessation of coronary flow and angiographic visualization of vessel obstruction. In that study, restoration of flow was followed in subsequent angiographic frames by the passage of a

Cambridge Books Online © Cambridge University Press, 2009



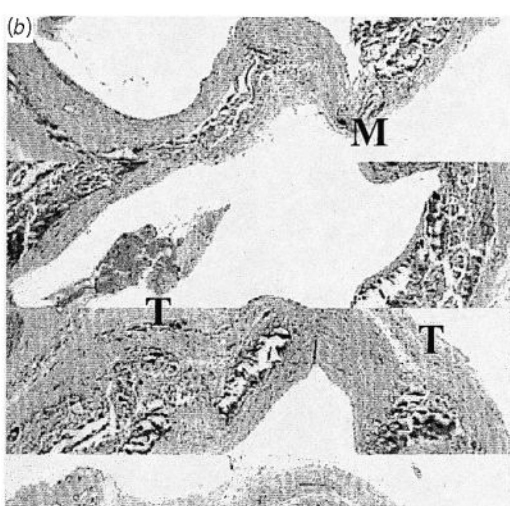


Fig. 33.2. (*a*) Histological examination of coronary arteries obtained from the Folts' model when coronary flow reached its nadir, shows that the lumen of the coronary artery is occluded by a platelet/fibrin rich thrombus (T). M indicates the medial layer. (*b*) Histological examination of coronary arteries obtained when coronary flow was normal shows that the artery lumen is clear, even if traces of the thrombus are still visible (T).

radiopaque material downstream from the stenosis. Additional and perhaps more convincing evidence for platelet-rich thrombi as the main cause of CFRs comes from a study by Golino et al. in which the authors have labelled endogenous platelets with ^{111}In: it could be shown that platelet accumulation at the site of the stenosis was maximal when coronary flow reached its nadir, while little accumulation was evident when the vessel was widely clear[11].

Although it has been demonstrated that the primary cause of CFRs is platelet aggregation at the site of stenosis, it is possible that local vasoconstriction, mediated by release of chemical mediators released by activated platelets, might contribute to the cyclic reductions in blood flow observed in this model, particularly when the endothelium is altered or absent. By means of ultrasonic crystals implanted immediately distal to the stenosis and 1–2 cm below, Golino et al. have demonstrated the occurrence of a marked coronary vasoconstriction at the site of the stenosis, i.e. where the endothelium had been previously damaged and where platelets and platelet-derived substances accumulate maximally in the arterial wall[12]. On the contrary, the vasoconstriction observed at the site more distal to the stenosis, i. e. where special attention was paid to maintain the endothelium intact, was much less severe and not different from that observed during a mechanical external occlusion of the coronary artery[12]. It is of note that the marked vasoconstriction observed at the site of the stenosis could be completely prevented by administration of LY53857, a selective serotonin receptor antagonist, or SQ29548, a selective TxA_2 receptor antagonist[12], but not by nitroglycerin or diltiazem[13], thus indicating a prominent role of serotonin and TxA_2 in causing this platelet-related coronary vasoconstriction.

Pathophysiological and pharmacological observations

Initial studies have demonstrated that TxA_2 represents an important mediator of CFVs: interventions that interfere with the synthesis of this prostaglandin, such as aspirin or dazoxiben, a selective TxA_2 synthase inhibitor, or with the receptor of TxA_2, such as SQ 29548, usually result in elimination of CFVs in about 70% of the treated animals[2,6,8,9]. Furthermore, plasma concentrations of TxB_2 in the blood obtained distally from the stenosis are elevated during CFVs, while they return to control values after abolition of CFVs[8].

The observation that interventions that interfere with the synthesis and/or the receptors of TxA_2 resulted in elimination of CFVs in only 70% of the animals suggested that other chemical mediators are involved in initiating and/or sustaining CFVs. It was later found that serotonin concentrations (another chemical mediator released upon platelet activation) at the site of the stenosis increase significantly during CFVs[14] and that the administration of different serotonin receptor antagonists also results in elimination of CFVs in the majority of the animals[14–16].

Cambridge Books Online © Cambridge University Press, 2009

Administration of serotonin receptor antagonists abolished CFVs virtually in all animals that did not initially respond to TxA_2 inhibitors[16]. Thus, TxA_2 and serotonin play an important role in promoting and sustaining platelet aggregation and thrombus formation at sites of coronary artery stenosis and endothelial injury. Later studies have then demonstrated that TxA_2 and serotonin are not the only mediators of CFRs, as other substances are involved in the formation of intravascular thrombi under conditions of arterial stenosis and endothelial injury, like those found in this experimental model, including ADP[17], PAF[18], and oxygen free radicals[19].

It was initially believed that this model was exclusively platelet dependent; however, the importance of activation of the coagulation cascade in this model has been later emphasized, as thrombin inhibitors resulted in elimination of CFRs in the vast majority of the animals treated[20]. In addition, more recent studies have shown the importance of tissue factor-dependent activation of the coagulation cascade in initiating and/or sustaining CFRs in this model[21–23]. Finally, it should be mentioned that intravascular thrombi formation may be favoured not only by the presence of activating chemical mediators, but also by the relative lack of inhibiting substances, such as EDRF[24] or tissue factor pathway inhibitor[25].

Applicability to different arteries and to other species

In recent years several investigators, including ourselves, have shown that the same combination of severe vessel narrowing and de-endothelialization results in CFRs in arteries other than the coronary. CFRs have been elicited in femoral arteries in anesthetized dogs with similar degrees of vessel narrowing and deliberate denudation of the artery. Folts et al.[6] have demonstrated that CFRs can be produced in awake dogs with chronically implanted coronary constrictors and flow probes. CFRs were prevented in the interim between implantation and acute study by the administration of aspirin. Al-Wathiqui[26] and Gallagher[27] and coworkers have demonstrated that progressive carotid or coronary arterial narrowing with ameroid constrictors will result in CFRs days to weeks after surgical implantation. These dogs apparently did not undergo deliberate vessel denudation at the time of implantation. Perhaps focal inflammation developed in the intervening week (or weeks) between surgery and the development of CFRs in these animals. CFRs have also been elicited in the renal[28] and carotid[29] arteries of cynomologus monkeys and in rabbit carotid arteries[30]; the latter species has been extensively utilized in our laboratory[18,21,31–34].

Recently, Eidt et al.[35] showed that conscious dogs equipped with the same constrictors over segments of the LAD showing endothelial injury undergo repetitive CFRs in response to exercise, but not ventricular pacing. The frequency and severity of CFRs varied more in this model. Some CFRs were non-occlusive. CFRs of most dogs eventually deteriorated to persistent no- or low-flow states. Unlike the open chest preparation, flow restorations observed in this model occurred spontaneously, probably because the severity of the stenosis produced by Eidt et al.[35] was not as great as that produced by most practitioners of the Folts' model, as reflected by the ability of CBF to increase above control levels initially during exercise.

Advantages and disadvantages

The Folts' model has several advantages that make it very attractive for investigators in the field of intravascular thrombosis. Perhaps the most important attractive feature of this model is that it closely mimics the clinical situation of acute coronary syndromes, characterized by plaque rupture and arterial stenosis. In this respect, the Folts' model of coronary thrombosis should be considered 'more physiologic' than other models like those described below. Another advantage of the Folts' model is its amenability to dose–response studies. Unlike other models in which the thrombotic processes are dynamic, occurring over several minutes to hours, CFRs in the Folts' model are repetitive and unchanging. In the many dogs that received either no intervention or vehicle after initiating CFRs, flow patterns usually remain unchanged for at least several hours. Thus, one can evaluate several doses of an investigational drug in a single dog, and this points out another feature of the model: its amenability to quantification of drug response.

The most important disadvantage of the Folts' model is that we are dealing with a healthy artery, as opposed to atherosclerotic arteries (which undergo rupture or ulceration at a site of a pre-existing atherosclerotic plaque) in the clinical scenario. This might, in theory, result in different mechanisms of thrombus formation, as well as in different responses to various drugs. For example, flow restorations in the Folts' model require vigorous shaking, while in unstable angina the thrombus is usually not persistent, and only occasionally can lead to a persistent and total thrombotic coronary occlusion. However, if these (few) limitations are taken into proper consideration, the Folts' model of thrombus formation is a well-established method to study the pathophysiology of platelet-coagulation factors/vessel wall interactions, as well as to determine the pharmacology of antithrombotic agents.

Cambridge Books Online © Cambridge University Press, 2009

Copper coil model of arterial thrombosis

In the mid-1960s, Blair et al.[36] reported on the ability of spiral wires constructed of aluminum/magnesium alloy inserted into the coronary circulation of dogs to produce slowly developing occlusive thrombi. In this early description of the technique, the spiral was inserted through the coronary vessel wall in open-chest dogs. Several years later, Kordenat et al.[37] described a modification of this model, the salient feature being insertion of the thrombogenic coils via the left carotid artery under fluoroscopic guidance in closed-chest dogs. Later studies by Cercek et al.[38] and Bergmann[39] and coworkers used this basic experimental method for producing arterial thrombi to evaluate thrombolysis and the utility of reperfusion.

As described by Kordenat et al.[37] and Bergmann et al.[39], a thrombogenic coil is advanced via the left carotid artery into a coronary artery with the aid of a guiding catheter and a smaller inner catheter or guidewire in closed-chest dogs. Occlusive thrombosis, heralded by electrocardiographic signs of ischemia and confirmed by the development of a filling defect distal to the coil, occurs almost invariably in the animals in which the coil is implanted. Ventricular fibrillation occurs in approximately one of five dogs undergoing coronary thrombosis, but most of these dogs can be converted to sinus rhythm by DC countershock. A thrombotic occlusion consistently occurs within minutes to hours, depending on the composition (alloy) and size of the coil after insertion, following a flow pattern that suggests gradual thrombus growth, leading in turn to a progressively narrowing stenosis within the coil. Occasionally, spontaneous flow restorations similar to those seen in the Folts' and electrical stimulation models precede total occlusion, but this occurs infrequently.

Thrombi produced in the coil model are considered to be relatively fibrin-rich, on the basis of the model's pharmacologic profile. Clinically relevant doses of t-PA and streptokinase lyse these thrombi[39–41], and heparin prolongs (but does not prevent) original thrombosis[37,38]. However, histologic examination of thrombi removed from copper coils 15 mm after occlusive thrombus formation reveals a platelet-rich thrombus that also contains red blood cells and fibrin[42].

Certain methodologic factors can greatly influence the nature of thrombi in this model and thus may contribute significantly to the differences in the relative composition of the thrombus, and thus to different results between investigators. The speed with which the copper coil is inserted to its destination may have important consequences. Copper coils have been inserted into the LAD and femoral arteries[42]. Despite the longer distance, it takes less time to insert coils into the femoral artery compared with the LAD. Moreover, we have observed more varied and shorter average times to occlusion in the coronary model. Sometimes a filling defect is observed immediately after coil placement, due presumably to snow-ploughing by the coil of clotted blood adherent to the guidewire during its passage to the coronary artery.

Another technical aspect worth noting is the geometry and composition of the coil. Kordenat and Kezdi[37] observed times to occlusion exceeding 2 hours, with shorter coils containing fewer spirals. Aluminium and magnesium alloys appear to be less thrombogenic than copper coils, as signs of infarction did not appear until 24–48 h after placement of coils or spirals constructed of these metals[36,37].

Pathophysiological and pharmacological observations

In contrast to the Folts' model, the copper coil model has been used almost exclusively for the evaluation of either thrombolytic agents or adjunctive drugs[38–42]. The original description of this model for the study of thrombolytic agents and their influence on myocardial injury was by Bergmann et al.[41], who demonstrated that timely reperfusion with intracoronary streptokinase salvaged myocardium and metabolic function. Subsequent studies from that group with this model demonstrated for the first time the ability of t-PA to lyse thrombi in vivo[39].

Bush et al.[42] and van der Werf et al.[43] have demonstrated a dose–response with t-PA and prourokinase, respectively, in this model. In addition, several adjunctive compounds have been evaluated for their ability to improve the thrombolytic effects of t-PA and/or to prevent or delay reocclusion after discontinuation of thrombolytic therapy. For example, heparin[38], Abciximab[44,45], as well as selective serotonin and thromboxane receptor antagonists[40,46,47], all are associated with a potentiation of the thrombolytic properties of t-PA and a simultaneous delay of reocclusion following discontinuation of t-PA infusion.

Applicability to other arteries and to other species

Bush et al. recently described a femoral arterial version of this model in closed-chest, anesthetized dogs[42]. It uses the same thrombogenic coils, but does not entail fluoroscopy. With Doppler flow probes placed directly on the femoral artery just proximal to the site of thrombosis, it allows the exact moment of thrombosis and thrombolysis to be detected, and the extent of blood flow restoration after lysis to be monitored continuously and accurately. An attractive

Cambridge Books Online © Cambridge University Press, 2009

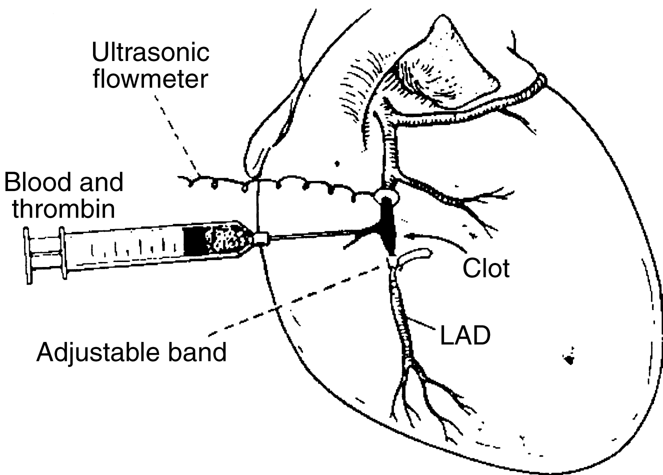


Fig. 33.3. Schematic representation of experimental preparation of a whole blood clot in canine left anterior descending artery (LAD) with a distal high-grade stenosis and a proximal ultrasonic flow probe (see text for details).

feature of this model is the ability to perform the procedure in smaller dogs, which may save expensive thrombolytic agents or investigational adjunctive drugs. Moreover, use of the femoral artery enables the model to be adapted to smaller animals, such as rabbits[47].

Advantages and disadvantages

One important disadvantage of this model is represented by the need for fluoroscopy to correctly position the coil in the coronary artery; this equipment is quite expensive and not all investigators have it at their disposal. Moreover, of all the models discussed in this review, the present one may be the least attractive from the standpoint of the thrombogenic stimulus resembling the human pathophysiology of myocardial infarction. However, it may be no less relevant to the human situation as the other three in its degree of thrombogenicity and response to thrombolytic agents. A particularly attractive feature of the model is the uniform time to original thrombus formation, response to thrombolytics, and reocclusion[38–48].

Thrombin-induced clot formation

A canine model of thrombin-induced clot formation was developed by Gold et al.[49] in which localized coronary thrombosis was produced in the LAD. This is a variation of the technique originally described by Collen et al.[50] who used radioactively labelled fibrinogen to monitor thrombolysis of rabbit jugular vein clots. The vessel was intentionally de-endothelialized by traumatization via external compression with blunt forceps. Snare occluders were then placed proximal and distal to the damaged site, and thrombin (10 U) was injected into the isolated LAD segment in a small volume via a previously isolated side branch. Autologous blood (0.3–0.4 ml) mixed with calcium chloride (0.05 M) also was injected into the isolated LAD segment, producing a stasis-type red clot superimposed on an injured blood vessel. The snares were released 2–5 min later and total occlusion was confirmed by selective coronary angiography (Fig. 33.3).

This model of coronary artery thrombosis relies on the conversion of fibrinogen to fibrin by thrombin. The fibrin-rich thrombus contains platelets, but at no greater concentration than in a similar volume of whole blood. Once the thrombus is formed, it is allowed to age for 1–2 hours, after which a thrombolytic agent can be administered to lyse the thrombus and restore blood flow.

Pathophysiological and pharmacological observations

In the initial study described by Gold et al.[49] in 1984, recombinant t-PA was characterized for its ability to lyse 2-hour-old thrombi. Tissue plasminogen activator was infused at doses of 4.3, 10, and 25/kg/min, i.v., and resulted in reperfusion times of 40, 31, and 13 min, respectively. Thus, in this model of canine coronary thrombosis, t-PA exhibited dose-dependent coronary thrombolysis; it has been also demonstrated that streptokinase also elicited dose-dependent thrombolysis in this model. Furthermore, it is possible to study the effect of different doses of t-PA on parameters of systemic fibrinolytic activation, such as fibrinogen, plasminogen, and α_2-antiplasmin as well as to assess myocardial infarct size.

Subsequently, Gold et al.[44,52] modified the model to study not only reperfusion, but also acute reocclusion. Clinically, reocclusion is a persistent problem after effective coronary thrombolysis, which is reported to occur in 15–45% of patients. Thus, an animal model of coronary reperfusion and reocclusion would be important from the standpoint of evaluating adjunctive therapies to t-PA to hasten and/or increase the response rate to thrombolysis as well as prevent acute reocclusion.

The model of thrombin-induced clot formation in the canine coronary artery was modified such that a controlled high-grade stenosis was produced with an external constrictor. Blood flow was monitored with an electromagnetic flow probe. In this model of clot formation with superimposed stenosis, reperfusion in response to t-PA

Cambridge Books Online © Cambridge University Press, 2009

occurs with subsequent reocclusion[44]. The monoclonal antibody against the human GPIIb/IIIa receptor developed by Coller et al.[29] and tested in combination with t-PA in the canine thrombosis model hastened t-PA-induced thrombolysis and prevented acute reocclusion[44,45]. These actions in vivo were accompanied by abolition of ADP-induced platelet aggregation and markedly prolonged bleeding time.

Applicability to other arteries and to other species

Localized thrombosis can also be produced in rabbit peripheral blood vessels such as the femoral artery by injection of thrombin, calcium chloride, and fresh blood via a side branch[53]. The advantage of using a rabbit femoral artery instead of a dog coronary artery relates to the availability of test compounds under study. On a per kilogram basis, 5–10 times less compound will be required in the rabbit, as opposed to the dog.

Either femoral artery is isolated distal to the inguinal ligament and traumatized distally from the lateral circumflex artery by rubbing the artery with blunt forceps. An electromagnetic flow probe is placed distal to the lateral circumflex artery to monitor femoral artery blood flow. The superficial epigastric artery is cannulated for induction of the thrombus: localized thrombi distal to the lateral circumflex artery with snares approximately 1 cm apart are induced by the sequential injection of thrombin, $CaCl_2$ (1.25 mmol), and a volume of blood sufficient to distend the artery. After 30 mm the snares are released and femoral blood flow is monitored for 30 mm to confirm total obstruction of flow by the thrombus.

After establishing a fully occlusive thrombus, t-PA can be administered alone or in combination with various other agents. At doses of 5.0, 7.5, and 10 μg/kg/min i.v. for 90 mm, t-PA caused reperfusion in 30, 50, and 100% of animals, respectively[45], although the times to restoration of flow did not differ between doses, occurring between 60 and 70 min.

Advantages and disadvantages

These models are not appropriate for evaluating drugs for their ability to inhibit original thrombosis. The model does lend itself well to the evaluation of both thrombolytic agents and adjunctive therapies, e.g. for their ability to hasten and/or enhance lysis or prevent acute reocclusion after discontinuing administration of the thrombolytic drug.

Electrically induced coronary thrombosis

An interesting technique for inducing coronary artery thrombosis was described by Salazar[54] in 1961; in this model anodal current was delivered to the intravascular lumen of a coronary artery in the dog via a stainless steel electrode. The electrode was positioned under fluoroscopic control, which somewhat complicated the procedure. Subsequently, Romson et al.[55] modified the procedure such that the electrode was placed directly into the coronary artery of an open-chest, anesthetized dog. This technique then allows one to produce a thrombus in the anesthetized animal or to close the chest after inserting the electrode and allow the animal to recover, after which thrombosis can be elicited in the conscious state. The advantage of this modification is that it allows induction of thrombus formation without the need for fluoroscopy.

The stimulation electrode may be constructed from a 25–26-gauge stainless steel hypodermic needle tip, which is attached to a 30-gauge teflon-insulated, silver-coated copper wire. Anodal current is delivered to the electrode via either a 9 V nickel-cadmium battery with the anode connected in series to a 250 000 ohm potentiometer or with a Grass stimulator connected to a Grass constant current unit and a stimulus isolation unit. The cathode in both cases is placed into a subcutaneous site completing the circuit. The anodal current can be adjusted to deliver 50–200 μA. Anodal stimulation results in focal endothelial disruption, which in turn induces platelet adhesion and aggregation at the damaged site. This process is then followed by further platelet aggregation and consolidation with the growing thrombus entrapping red blood cells.

A modification of the method of Romson et al. involves placement onto the coronary of an external, adjustable occluder[56] to produce a fixed stenosis on the coronary artery. A flow probe to record coronary blood flow is placed on the proximal portion of the artery followed by the stimulation electrode, with the clamp being placed most distally (Fig. 33.4). The degree of stenosis can then be controlled by adjusting the clamp. The resulting stenosis is produced in an effort to mimic the human pathophysiology of atherosclerotic coronary artery disease, whereby thrombolytic therapy restores CBF through a coronary artery with residual narrowing due to atherosclerotic plaque formation.

Another modification of the electrical stimulation model that merits discussion is described by Benedict et al.[57] They discontinued anodal current when mean distal coronary flow velocity (measured with a Doppler flowmeter) increased by approximately 50%, reflecting disruption of normal axial flow by the growing thrombus. Occlusive

Cambridge Books Online © Cambridge University Press, 2009

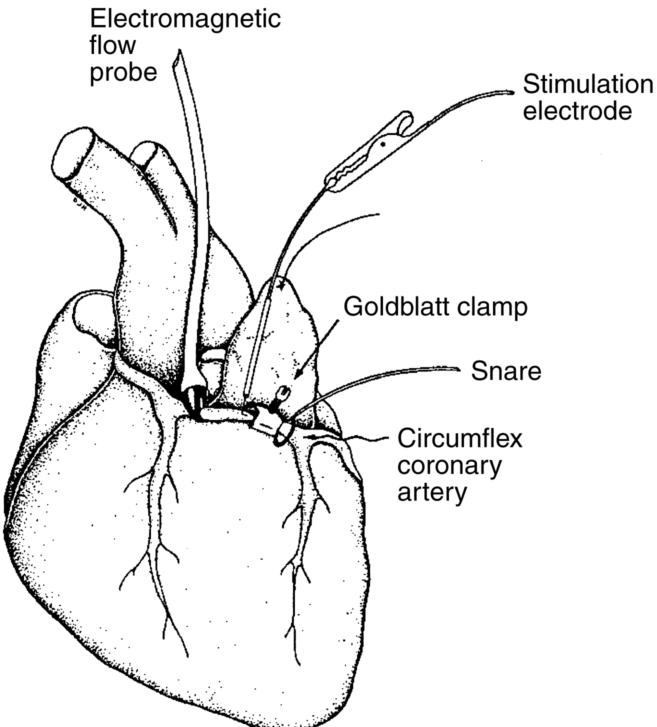


Fig. 33.4. Model of coronary artery thrombosis induced in the dog by electricity. Electrical injury to the intimal surface of the artery leads to occlusive thrombus formation. The thrombus is formed in the presence of a flow-limiting stenosis induced by a Goldblatt clamp (see text for details).

thrombosis occurred within 1 hour after stopping the current (2 hours after starting the current). In these studies coronary sinus plasma levels of serotonin, an index of intravascular platelet aggregation, were increased approximately 20-fold just before occlusive thrombus formation. The results of these studies agree with others in showing that either proximal flow velocity or electromagnetically measured CBF decline trivially over the majority of the time period in which the thrombus is growing. The largest declines in flow occur over a small and terminal fraction of the period between initial vessel perturbation and final occlusion. During that interval, coronary luminal area decreases rapidly and to a critical degree, as platelets accrue at the growing thrombus. The studies by Benedict et al.[57] show that this final phase of thrombosis can occur independently of electrical stimulation. This variation of the model may be attractive to those who wish occlusive thrombosis to occur without continued electrical stimulation.

Pathophysiological and pharmacological observations

Several studies evaluating anticoagulants, antithrombotic, and/or thrombolytic drugs have been performed using this model. In the initial report by Romson et al.[56], the cyclooxygenase inhibitor ibuprofen was evaluated. Comparison of myocardial infarct size, thrombus weight, arrhythmia development, and scanning electron microscopy of drug-treated and control animals indicated that ibuprofen protected against the deleterious effects of coronary artery thrombosis in the conscious dog. Subsequent studies in the same model and laboratory evaluated the antithrombotic potential of various TXA_2 synthetase inhibitors, such as U 63557a, CGS 13080, OKY 1581, and dazoxiben. When the TXA_2 synthetase inhibitors were administered before induction of the current, OKY 1581[58] and CGS 13080[59] reduced the incidence of coronary thrombosis, whereas U 63557A[60] and dazoxiben[61] were ineffective and partially effective, respectively. The differences in efficacy noted among the TXA_2 synthetase inhibitors were ascribed to differences in potency and duration of action.

Other investigators have used this model to study the prevention of original coronary thrombosis in the dog. FitzGerald et al.[62] studied the combination of the TXA_2 synthetase inhibitor, U 63557A, alone or in combination with L-636 499, an endoperoxide/thromboxane receptor antagonist. U 63557A alone did not prevent non-specific coronary thrombosis when administered before current application, whereas the combination of U 63557A and L-636 499 was highly effective. These data suggest that prostaglandin endoperoxides may modulate the effects of TXA_2 synthetase inhibitors and that this response may be blocked by concurrent administration of an endoperoxide/thromboxane receptor antagonist. The murine monoclonal antibody to the platelet GPIIb/IIIa (7E3, Abciximab) was studied in this model for its ability to prevent thrombus formation. At a dose of 0.8 mg/kg i.v., the 7E3 monoclonal antibody completely prevented original thrombus formation[63].

In addition to evaluation of antithrombotic agents, the electrical injury model is useful for studying thrombolytic drugs. Typically, the thrombus is allowed to form without drug intervention and then aged for various periods of time. Schumaker et al.[56] demonstrated that intracoronary streptokinase was an effective thrombolytic drug in this model, the thrombolytic effectiveness being augmented by the concurrent administration of heparin and prostacyclin, or by a TXA_2 synthetase inhibitor[56]. In other studies, Shebuski et al.[64] demonstrated that the TXA_2 receptor antagonist, BM 13.177, hastened t-PA-induced thromboly-

Cambridge Books Online © Cambridge University Press, 2009

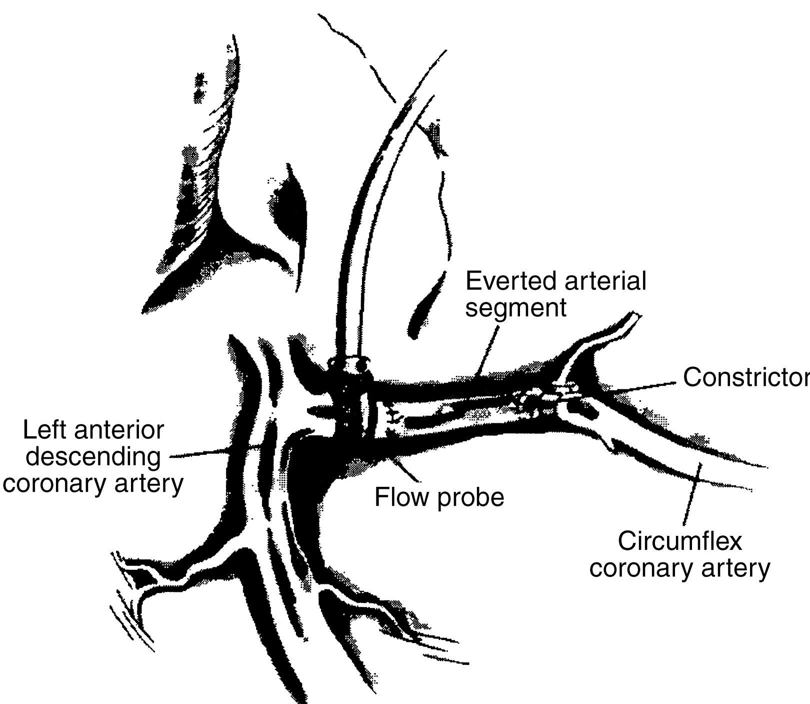


Fig. 33.5. Schematic representation of experimental preparation of a platelet-rich thrombus in canine everted circumflex coronary artery segment graft with a distal high grade stenosis and a proximal ultrasonic flow probe (see text for details).

sis and prevented acute thrombotic reocclusion. Van der Giessen et al.[66] subsequently demonstrated that BM 13.177 prevented original thrombus formation in 75% of pigs undergoing electrical stimulation; aspirin was ineffective in this porcine model[65].

These and other studies underscore the potential for adjunctive therapy to accelerate thrombolysis and/or prevent reocclusion, both contributing to greater salvage of ischemic myocardium.

Advantages and disadvantages

One important advantage of the electrically induced coronary thrombus model is represented by its versatility: original thrombosis responds to antithrombotic agents; furthermore, after establishment of an occlusive thrombus and administration of thrombolytic agents, it develops spontaneous reocclusion, making it amenable to evaluation of potentially adjunctive agents. Finally, as is true for the coil and thrombin injection models, the electrically induced thrombus model may be used to produce and study myocardial infarction. One potential disadvantage is the lack of similarity with the pathophysiology of arterial thrombosis in patients.

Arterial eversion graft model

The model is schematically represented in Fig. 33.5. Mongrel dogs are anesthetized with intravenous sodium pentobarbital, intubated, and artificially ventilated. A left thoracotomy is performed, and the internal mammary artery is cannulated for continuous blood pressure monitoring. An ultrasonic blood flowmeter is placed around the circumflex coronary artery proximal to the site of the eversion graft. A stenosis is then produced with a 2 mm-wide plastic wire tie placed around the coronary artery distal to the eversion graft and constricted to reduce the blood flow to about 40% of baseline, which corresponds to a more than 90% reduction in luminal diameter. The circumflex coronary artery is then dissected out of the epicardium, and a 1 cm segment is excised between two microvascular clamps, stripped of excessive adventitial tissue, everted inside-out, and reinserted by end-to-end anastomosis using 8 to 12 interrupted sutures with 7–0 nylon. The microvascular clamps occluding the proximal and distal ends of the transsected artery are then released. The surgical procedure is carried out without an extracorporeal circulation device. Coronary angiography is performed by manual injection of 1–2 ml Renografin 76 through a

Cambridge Books Online © Cambridge University Press, 2009

modified Judkin's 7F left Amplatz catheter inserted from a carotid artery. After release of the vessel clamps, spontaneous persistent occlusion of the everted graft segment occurs in approximately 80% of the dogs within an average of 5 minutes, as demonstrated with the flow probe and confirmed by angiography.

Intravenous administration of thrombolytic drugs is usually carried out when the thrombus is demonstrated to be persistent, i.e. after a complete and stable occlusion is obtained for 30 minutes. After reperfusion has occurred, blood flow is continuously monitored for evidence of reocclusion, with the final confirmation by angiography using the same criteria as for establishing reperfusion.

Pathophysiological and pharmacological observations

The efficacy of thrombolytic agents, including streptokinase, urokinase, and t-PA, were evaluated in this model and compared with their efficacy in the absence of a superimposed stenosis. In the absence of stenosis reperfusion times were similar to animals with a superimposed stenosis; spontaneous reocclusion did not occur within 2 hours after the end of the infusion in the absence of restenosis[66]. Adjunctive therapies, represented by aspirin, Argatroban, or Abciximab were all associated with a simultaneous decrease in reperfusion times and an increase in reocclusion times[67].

Applicability to other arteries and to other species

The arterial eversion graft model can be applied to rabbit femoral arteries. This model was originally developed by Hergrueter et al.[68] Briefly, New Zealand white rabbits are anesthetized with intramuscular injection of ketamine (35 mg/kg) and xylazine (5 mg/kg). A groin incision is made to expose the femoral artery between the inguinal ligament and the distal bifurcation. The epigastric artery is cannulated with Silastic tubing (0.012″ i.d.) for intraarterial administration of study drug. A 5 mm segment of the right femoral artery is excised between two ligations, stripped of excessive adventitial tissue, everted inside-out, and inserted into the left femoral artery under a surgical microscope by end-to-end anastomosis using 12 uninterrupted sutures with 10–0 nylon. The microvascular clamp that clamps off the proximal and distal ends of the transsected artery is released.

Blood flow in the left femoral artery containing the everted graft is continuously monitored with an ultrasonic flowmeter. Thirty minutes after occlusion, study drugs can be administered via the cannulated femoral vein (intravenous route) or superficial epigastric artery (intra-arterial route) by either bolus injections or continuous infusion.

Advantages and disadvantages

The main disadvantage of the arterial eversion graft model is that it requires significant surgical skills to be performed. Additionally, for the rabbit femoral model, a surgical microscope is required. Furthermore, as with the copper coil and the electrical injury models, the thrombogenic stimulus may be considered rather 'unphysiologic'.

Conclusions

We have described four models of arterial thrombosis and/or thrombolysis that allow evaluation of antithrombotic drugs alone or in combination with thrombolytic agents. The experimental animal preparations described in this review were not designed to mimic the pathophysiology of human arterial thrombosis, but instead are models of intravascular coagulation induced by different stimuli. Each model possesses distinct characteristics with regard to a number of important variables, including relative contribution by platelets and fibrin and the speed with which occlusive thrombosis occurs after initiation of the thrombogenic stimulus (or stimuli). For instance, thrombi forming in the Folts' model of de-endothelialization combined with severe stenosis probably are the most platelet rich, whereas those produced in the Gold thrombin injection model are relatively platelet poor. The other models probably lie within these two extremes, as the thrombus contains both platelets and fibrin.

Considering the uncertainties about the relative roles of coagulation vs. platelets in patients undergoing myocardial infarction, unstable angina, or acute coronary reocclusion after thrombolysis, it would be inappropriate to speculate as to which of these models is the most clinically relevant. The prospect for advancing the clinical relevance of animal models of thrombosis is further complicated by the likely variability in the causes of thrombosis in patients: atherosclerotic human coronary arteries undergoing plaque fissure and/or hemorrhage probably differ substantially from the arterial surfaces provided in these models, despite attempts to produce some degree of de-endothelialization and/or vessel narrowing.

If put in the proper perspective, the data obtained in these (and perhaps other) models of intravascular thrombosis may allow the investigator to identify and compare novel therapies. Animal models provide an important link between test tube assays of platelet aggregation, coagula-

Cambridge Books Online © Cambridge University Press, 2009

tion, or fibrinolysis and clinical evaluation in humans. In this process, important information is generated, not the least of which is an answer to the question, 'does it work?' In addition, dosage, duration of action, and some idea about safety can be gleaned from studies of these animal models.

The ultimate model is humans and the ultimate evaluation is a well-defined population of patients. It should be borne in mind that t-PA was evaluated in fewer than a dozen dogs in one arterial thrombosis model (coronary copper coil) before clinical testing in humans was begun. Thus, one probably does not need to evaluate a new antithrombotic or thrombolytic agent in all four models. We would hope that knowledge and availability of several models will allow informed decisions as to which model (or models) to evaluate novel antithrombotic or thrombolytic agents before initiating expensive clinical trials.

References

1 Cohen C. The case for the use of animals in biomedical research. *N Engl J Med* 1986; 315: 865–870.
2 Folts J D, Crowell EB, Rowe GG. Platelet aggregation in partially obstructed vessels and its elimination with aspirin. *Circulation* 1976; 54: 365–370.
3 Willerson JT, Golino P, Eidt J, Campbell W, Buja L. Specific platelet mediators and unstable coronary artery lesions. *Circulation* 1989; 80: 198–205.
4 Strony J, Beaudoin A, Brands D, Adelman B. Analysis of shear stress and hemodynamic factors in a model of coronary artery stenosis and thrombosis. *Am J Physiol* 1993; 265: H1787-H1796.
5 Aiken JW, Gorman RR, Shebuski RJ. Prevention of blockage of partially obstructed coronary arteries with prostacyclin correlates with inhibition of platelet aggregation. *Prostaglandins* 1979; 17: 483–494.
6 Folts JD, Gallagher K, Rowe GG. Blood flow reductions in stenosed canine coronary arteries: vasospasm or platelet aggregation. *Circulation* 1982; 65: 248–255.
7 Aiken JW, Shebuski RJ, Miller OV, Gorman RR. Endogenous prostacyclin contributes to the efficacy of a thromboxane synthetase inhibitor for preventing coronary artery thrombosis. *J Pharmacol Exp Ther* 1981; 219: 299–308.
8 Bush LR, Campbell WB, Buja LM, Tilton GD, Willerson JT. Effects of the selective thromboxane synthetase inhibitor dazoxiben on variations in coronary blood flow in stenosed canine coronary arteries. *Circulation* 1984; 69: 1161–1170.
9 Ashton JH, Schmitz JM, Campbell WB et al. Inhibition of cyclic flow variations in stenosed canine coronary arteries by thromboxane A2/prostaglandin H2 receptor antagonists. *Circ Res* 1986; 59: 568–578.
10 Ashton JH, Benedict CR, Fitzgerald C et al. Serotonin is a mediator of cyclic flow variations in stenosed canine coronary arteries. *Circulation* 1986; 73: 572–578.
11 Golino P, Buja LM, Ashtom JH, Kulkarni P, Taylor A, Willerson JT. Effect of thromboxane and serotonin receptor antagonists on intracoronary platelet deposition in dogs with experimentally stenosed coronary arteries. *Circulation* 1988; 78: 701–711.
12 Golino P, Ashton JH, Buja LM et al. Local platelet activation causes vasoconstriction of large epicardial canine coronary arteries in vivo. Thromboxane A_2 and serotonin are possible mediators. *Circulation* 1989; 79: 154–166.
13 Golino P, Buja LM, Yao SK, McNatt J, Willerson JT. Failure of nitroglycerin and diltiazem to reduce platelet-mediated vasoconstriction in dogs with coronary artery stenosis and endothelial injury: further evidence for thromboxane A_2 and serotonin as mediators of coronary artery vasoconstriction in vivo. *J Am Coll Cardiol* 1990; 15: 718–726.
14 Ashton JH, Benedict CR, Fitzgerald C et al. Serotonin is a mediator of cyclic flow variations in stenosed canine coronary arteries. *Circulation* 1986; 73: 572–578.
15 Bush LR, Campbell WB, Kern K et al. The effects of $alpha_2$ adrenergic and serotonergic receptor antagonists on cyclic blood flow alterations in stenosed canine coronary arteries. *Circ Res* 1984; 55: 642–652.
16 Ashton JH, Ogletree ML, Michel IM et al. Cooperative mediation by serotonin S_2 and thromboxane A_2/prostaglandin H_2 receptor activation of cyclic flow variations in dogs with severe coronary artery stenoses. *Circulation* 1987; 76: 952–959.
17 Yao SK, Ober J, Benedict CR et al. ADP plays an important role in mediating platelet aggregation and cyclic flow variations in vivo in stenosed and endothelium-injured canine coronary arteries. *Circ Res* 1992; 70: 39–48.
18 Golino P, Ambrosio G, Ragni M et al. Short-term and long-term role of platelet activating factor as a mediator of in vivo platelet aggregation in rabbit carotid and canine coronary arteries with experimental stenosis and endothelial injury. *Circulation* 1993; 88: 1205–1212.
19 Yao SK, Ober JC, Gonenne A et al. Active oxygen species play a role in mediating platelet aggregation and cyclic flow variations in severely stenosed and endothelium-injured coronary arteries. *Circ Res* 1993; 73: 952–967.
20 Eidt JF, Allison P, Noble S et al. Thrombin is an important mediator of platelet aggregation in stenosed canine coronary arteries with endothelial injury. *J Clin Invest* 1989; 84: 18–25.
21 Pawashe AB, Golino P, Ambrosio G et al. A monoclonal antibody against rabbit tissue factor inhibits thrombus formation in stenotic injured rabbit carotid arteries. *Circ Res* 1994; 74: 56–63.
22 Ragni M, Cirillo P, Pascucci I et al. A monoclonal antibody against tissue factor shortens tissue-plasminogen activator lysis time and prevents reocclusion in a rabbit model of carotid artery thrombosis. *Circulation* 1996; 93: 1913.
23 Golino P, Ragni M, Cirillo P et al. Antithrombotic effects of recombinant human, active site-blocked factor VIIa in a rabbit model of recurrent arterial thrombosis. *Circ Res* 1998; 82: 39–46.

Cambridge Books Online © Cambridge University Press, 2009

24 Golino P, Cappelli-Bigazzi M, Ambrosio G et al. Endothelium-derived relaxing factor modulates platelet aggregation in an *in vivo* model of recurrent platelet activation. *Circ Res* 1992; 71: 1447–1453.
25 Ragni M, Golino P, Cirillo P et al. Endogenous tissue factor pathway inhibitor modulates thrombus formation in an in vivo model of rabbit carotid artery stenosis and endothelial injury. *Circulation* 2000; 102: 113–120.
26 Al-Wathiqui M H, Hartman J C, Brooks HL, Gross CJ, Warltier DC. Cyclical carotid artery flow reduction in conscious dogs: effect of a new thromboxane receptor antagonist. *Am Heart J* 1988; 116: 1482–1487.
27 Gallagher KR, Folts JD, Rowe GG. Comparison of coronary arteriograms with direct measurements of stenosed coronary arteries in dogs. *Am Heart J* 1978; 95: 338–347.
28 Schumacher WA, Heran CL, Goldenberg HJ, Harris DN, Ogletree ML. Magnitude of thromboxane receptor antagonism necessary for antithrombotic activity in monkeys. *Am J Physiol* 1989; 256: H726–H734.
29 Coller BS, Folts JD, Scudder LE, Smith SR. Antithrombotic effect of a monoclonal antibody to the platelet glycoprotein IIb/IIIa receptor in an experimental animal model. *Blood* 1986; 68: 783–786.
30 Golino P, Ambrosio G, Pascucci I, Ragni M, Russolillo E, Chiariello M. Experimental carotid stenosis and endothelial injury in the rabbit: An *in vivo* model to study intravascular platelet aggregation. *Thromb Haemost* 1992; 67: 302–310.
31 Golino P, Ambrosio G, Gresele P et al. The in vivo antiplatelet effects of thromboxane A_2 synthase inhibitors are potentiated by simultaneous thromboxane A_2/prostaglandin H_2 receptor blockade. *J Pharmacol Exp Ther* 1993; 266: 511–519.
32 Golino P, Ragni M, Cirillo P et al. Aurintricarboxylic acid reduces platelet deposition in stenosed and endothelially injured rabbit carotid arteries more effectively than other antiplatelet interventions. *Thromb Haemost* 1995; 74: 974–980.
33 Ragni M, Cirillo P, Pascucci I et al. A monoclonal antibody against tissue factor shortens tissue-plasminogen activator lysis time and prevents reocclusion in a rabbit model of carotid artery thrombosis. *Circulation* 1996; 93: 1913–1920.
34 Golino P, Ambrosio G, Ragni M et al. Inhibition of leucocyte and platelet adhesion reduces neointimal hyperplasia after arterial injury. *Thromb Haemost* 1997; 77: 783–791.
35 Eidt JF, Ashton J, Golino P, McNatt J, Buja LM, Willerson JT. Treadmill exercise promotes cyclic alterations in coronary blood flow in dogs with coronary artery stenoses and endothelial injury. *J Clin Invest* 1989; 34: 517–526.
36 Blair E, Nygren E, Cowley RA. A spiral wire technique for producing gradually occlusive coronary thrombosis. *J Thorac Cardiovasc Surg* 1964; 48: 476–485.
37 Kordenat RK, Kezdi P. Experimental intracoronary thrombosis and selective *in situ* lysis by catheter technique. *Am Heart J* 1972; 360–364.
38 Cercek B, Lew AS, Hod H et al. Enhancement of thrombolysis with tissue-type plasminogen activator by pretreatment with heparin. *Am J Cardiol* 1986; 74: 583–587.
39 Bergmann SR, Fox KAA, Ter-Pogossian MM, Sobel BE, Collen D. Clot-selective coronary thrombolysis with tissue-type plasminogen activator. *Science* 1983; 122: 1181–1183.
40 Golino P, Ashton JH, Glas-Greenwalt P, McNatt J, Buja LM, Willerson JT. Mediation of reocclusion by thromboxane A2 and serotonin after thrombolysis with tissue-type plasminogen activator in a canine preparation of coronary thrombosis. *Circulation* 1988; 77: 678–684.
41 Bergmann SR, Lereb RA, Ludbrook PA, Welch MJ, Ter-Pogossian MM, Sobel BF. Temporal dependence of beneficial effect of coronary thrombolysis characterized by positron tomography. *Am J Med* 1982; 73: 573–581.
42 Bush LR, Mellott MJ, Kanovsky SM, Holahan MA, Patrick DH. A model of femoral artery thrombolysis in dogs. *Fibrinolysis* 1989; 3: 107–114.
43 Van der Werf F, Jang IK, Collen D. Thrombolysis with recombinant human single chain urokinase-type plasminogen activator (rscu-PA): dose–response in dogs with coronary artery thrombosis. *J Cardiovasc Pharmacol* 1987; 9: 91–93.
44 Gold HK, Coller BS, Yasuda T et al. Rapid and sustained coronary artery recanilization with combined bolus injection of recombinant tissue-type plasminogen activator and monoclonal antiplatelet GpIIb/IIIa antibody in a canine preparation. *Circulation* 1988; 77: 670–677.
45 Yasuda T, Gold HK, Fallon JT et al. Monoclonal antibody against the platelet glycoprotein (GP) IIb/IIIa receptor prevents coronary artery reocclusion after reperfusion with recombinant tissue type plasminogen activator. *J Clin Invest* 1988; 81: 1284–1291.
46 Golino P, Ashton JH, McNatt J et al. Simultaneous administration of thromboxane A_2 and serotonin receptor antagonists markedly enhances thrombolysis and prevents or delays reocclusion after tissue-type plasminogen activator in a canine model of coronary thrombosis. *Circulation* 1989; 79: 911–918.
47 Golino P, Rosolowski M, Sheng-Kun Y et al. Endogenous prostaglandin endoperoxides and prostacyclin modulate the thrombolytic activity of tissue plasminogen activator. *J Clin Invest* 1990; 86: 1095–1102.
48 Marsh-Leidy E, Stern A M, Friedman PA, Bush LR. Enhanced thrombolysis by a factor IIIa inhibitor in a rabbit model of femoral artery thrombosis. *Thromb Res* 1990; 59: 15–26.
49 Gold HK, Fallon JT, Yasuda T et al. Coronary thrombolysis with recombinant human tissue-type plasminogen activator. *Circulation* 1984; 70: 700–707.
50 Collen D, Stassen JM, Verestraete M. Thrombolysis with human extrinsic (tissue-type) plasminogen activator in rabbits with experimental jugular vein thrombosis. *J Clin Invest* 1983; 71: 368–376.
51 Kopia GA, Kopaciewicz LJ, Ruffolo RR. Coronary thrombolysis with intravenous streptokinase in the anesthetized dog: a dose-response study. *J Pharmacol Exp Ther* 1988; 244: 956–962.
52 Gold HK, Leinbach RC, Garabedian HD et al. Acute coronary reocclusion after thrombolysis with recombinant human

Cambridge Books Online © Cambridge University Press, 2009

tissue-type plasminogen activator: prevention by a maintenance infusion. *Circulation* 1986; 73: 347–352.
53 Shebuski RJ, Storer BL, Fujita T. Effect of thromboxane synthetase inhibition on the thrombolytic action of tissue-type plasminogen activator in a rabbit model of peripheral arterial thrombosis. *Thromb Res* 1988; 52: 381–392.
54 Salazar AE. Experimental myocardial infarction, induction of coronary thrombosis in the intact closed-chest dog. *Circ Res* 1961; 9: 135–136.
55 Romson JL, Haack DW, Lucehesi BR. Electrical induction of coronary artery thrombosis in the ambulatory canine: a model for in vivo evaluation of antithrombotic agents. *Thromb Res* 1980; 17: 841–853.
56 Schumacher WA, Lee EC, Lucchesi BR. Augmentation of streptokinase-induced thrombolysis by heparin and prostacyclin. *J Cardiovasc Pharmacol* 1985; 7: 739
57 Benedict CR, Matthew B, Rex KA, Cartwright J, Jr., Sordahl LA. Correlation of plasma serotonin changes with platelet aggregation in an in vivo dog model of spontaneous occlusive coronary thrombus formation. *Circ Res* 1986; 58: 58–67.
58 Shea MJ, Driscoll EM, Romson JL, Pitt B, Lucchesi BR. Effects of OKY 1581, a thromboxane synthetase inhibitor, on coronary thrombosis in the conscious dog. *Eur J Pharmacol* 1984; 105: 285–291.
59 Simpson PJ, Smith JB, Jr., Rosenthal G, Lucchesi BR. Reduction in the incidence of tis by the thromboxane synthetase inhibitor CGS 13080 in a canine model of coronary artery injury. *J Pharmacol Exp Ther* 1986; 238: 497–501.
60 Hook BG, Schumacher WA, Lee DL, Jolly SR, Lucchesi BR. Experimental coronary artery thrombosis in thromboxane A_2 synthesis: evidence for alternate pathways for coronary thrombosis. *J Cardiovasc Pharmacol* 1985; 7: 174–181.
61 Schumacher WA, Lucehesi BR. Effect of the thromboxane synthetase inhibitor UK 37,248 (Dazoxiben) upon platelet aggregation, coronary artery thrombosis and vascular reactivity. *J Pharmacol Exp Ther* 1983; 227: 790–796.
62 FitzGerald DJ, Fragetta J, FitzGerald GA. Prostaglandin endoperoxides modulate the response to thromboxane synthetase inhibition during coronary thrombosis. *J Clin Invest* 1988; 82: 1708–1713.
63 Mickelson JK, Simpson PJ, Lucchesi BR. Antiplatelet monoclonal $F(ab')_2$ antibody directed against the platelet GPIIb/IIIa receptor complex prevents coronary artery thrombosis in the canine heart. *J Mol Cell Cardiol* 1989; 21: 393–405.
64 Shebuski RJ, Smith JM, Storer BL, Granett JR, Bugeiski PJ. Influence of selective endoperoxide/thromboxane A_2 receptor antagonism with sulotroban on lysis time and reocclusion rate after tissue plasminogen activator-induced coronary thrombolysis in the dog. *J Pharmacol Exp Ther* 1988; 246: 790–796.
65 Van der Giessen WJ, Zijlstra FJ, Berk L, Verdouw PD. The effect of the thromboxane receptor antagonist BM 13,177 on experimentally induced coronary artery thrombosis in the pig. *Eur J Pharmacol* 1988; 147: 241–248.
66 Yasuda T, Gold HK, Fallon JT et al. A canine model of coronary artery thrombosis with superimposed high grade stenosis for the investigation of rethrombosis after thrombolysis. *J Am Coll Cardiol* 1989; 13: 1409–1414.
67 Yasuda T, Gold HK, Yaoita H et al. Comparative effects of aspirin, a synthetic thrombin inhibitor, and a monoclonal antiplatelet GPIIb/IIIa antibody on coronary artery reperfusion, reocclusion and bleeding with tissue plasminogen activator. *J Am Coll Cardiol* 1990; 16: 714–722.
68 Hergrueter CA, Handren J, Kash R, May JW. Human recombinant tissue-type plasminogen activator and its effect on microvascular thrombosis in the rabbit. *Plast Reconstr Surg* 1988; 81: 418–424.

Cambridge Books Online © Cambridge University Press, 2009

Cambridge Books Online © Cambridge University Press, 2009

Part III

Pathology
A Hemostasis

Cambridge Books Online © Cambridge University Press, 2009

Cambridge Books Online © Cambridge University Press, 2009

Hereditary thrombocytopenias

Chris Van Geet[1,2], Kathleen Freson[2], Rita De Vos[3], and Jos Vermylen[2]

[1]Department of Paediatrics
[2]Centre for Molecular and Vascular Biology
[3]Morphology and Molecular Pathology, University Hospital, Leuven, Belgium

Although hereditary thrombocytopenia is overall a very rare disorder, many different forms have been well described. Inherited thrombocytopenia is frequently mistaken for an acquired platelet disorder. A careful medical and family history can generally allow differentiating congenital from acquired thrombocytopenia based on the duration of symptoms and the onset early in childhood. A detailed pedigree will often help to identify the inheritance pattern. The recognition of hereditary thrombocytopenia is important for avoiding potentially expensive and harmful treatments, as frequently administered in acquired chronic platelet disorders, and which will furthermore mostly be ineffective.

Some patients with hereditary thrombocytopenia have no clinical symptoms due to the moderate or mild thrombocytopenia and are most often detected by chance, due to the routine automated platelet counting and frequently associated automated mean platelet volume determination, when studying the peripheral blood. For this reason, probably more patients are being detected during recent years. It is only in the last few years that the underlying molecular abnormalities have become obvious. Due to the efforts of many research groups, the responsible gene, the defective gene product and the pathogenesis of many of these disorders have been unravelled. For many of these disorders, the clinical picture was well known and only recently the responsible gene and gene product were discovered. For others, the molecular mechanism was first unravelled in mice, and the human pathology was only discovered and defined by analogy with the animal model. Interestingly, discovering the molecular mechanisms of hereditary thrombocytopenia has led to a better understanding of the normal physiology of hematopoiesis.

Table 34.1 classifies the hereditary thrombocytopenias by their mode of inheritance and by the size of the platelets. The individual thrombocytopenia disorders will be discussed accordingly. Moreover, the possible association with qualitative platelet defects or with other clinical abnormalities will be discussed each time.

X-linked recessive thrombocytopenia

Microthrombocytopenia

The Wiskott–Aldrich syndrome (WAS)[1] is an X-linked recessive immunodeficiency disorder involving both B- and T- lymphocytes, and is characterized by eczema, increased susceptibility to infection and thrombocytopenic purpura with small, defective platelets. Patients are symptomatic at infancy with excessive bruising and often bloody diarrhea. The small dysmorphic platelets are destroyed by the spleen, thereby increasing the bleeding problem. Recurrent infections with encapsulated bacteria usually occur during the first year of life, as well as the atopic dermatitis. Therafter, often autoimmune cytopenia and/or vasculitis occurs. The high mortality is mostly due to infection, bleeding or malignancies, in particular lymphoreticular tumours and leukemias. WAS-patients have been treated by bone marrow or cord blood transplantation from an HLA-identical sibling or an HLA-matched unrelated donor, leading to a complete correction of the platelet and immunological abnormalities[2]. Patients who underwent splenectomy to control beeding, showed an increase in platelet count and had a rather good prognosis when prophylactically given antibiotics and intravenous immunoglobulins.

The mutant gene (*WASP*) responsible for this syndrome has been identified[3] and is mapped to Xp11.22–Xp11.23. *WASP* encodes a 502 amino acid protein (WASP) and is preferentially expressed in lymphocyte and megakaryocyte lineages. The gene product is very complex with well-defined

Table 34.1. Classification of hereditary thrombocytopenias

	X-linked recessive	Autosomal dominant	Autosomal recessive
Micro-thrombocytopenia	Wiskott–Aldrich syndrome *WASP* X-linked microthrombocytopenia *WASP*		
Macro-thrombocytopenia	X-linked macrothrombocytopenia *GATA1*	May-Hegglin anomaly *MYH9* Fechtner syndrome *MYH9* Sebastian platelet syndrome *MYH9* Epstein syndrome *MYH9?* Gray platelet syndrome *?* VWD 2B *VWF-gene* Platelet-type VWD *GPIbα -gene* 22q11 microdeletion *GPIbβ-deletion* Montreal platelet syndrome *Calpain defect?*	Bernard–Soulier syndrome *GPIbα -gene* *Or GPIbβ-gene* *Or GP IX-gene*
Thrombocytopenia with normal platelet size		Autosomal dominant form with normal platelet size *Locus on 10p* Paris–Trousseau thrombocytopenia *del11q23* Factor V Quebec *?* Familial platelet disorder with predisposition to acute myelogenous leukaemia (FPD/AML) *CBFA2*	Congenital amegakaryocytic thrombocytopenia *c-mpl gene* TAR-syndrome ?

Notes:
Genetic defect is indicated in italics.

regions that give the protein several faces[4] such as a pleckstrin homology domain, a GTPase binding domain and several proline-rich domains, typical for transcription factors. However, WASP does not reside in the nucleus, as would be expected for a transcription factor, but instead stays just beneath the cell surface, by binding of the pleckstrin homology domain to the phospholipids of the plasma membrane, thereby probably transducing incoming signals to the actin cytoskeleton. Furthermore, WASP binds with its GTPase binding domain to the active, GTP-bound Cdc42, a member of a superfamily of small GTP-hydrolysing proteins (GTP-ases). Cdc42 in its active form is involved in actin remodelling, such as formation of filopodia and the orientation of T-cells toward antigen-presenting cells. The precise function of WASP remains to be elucidated but is implicated through its different

Cambridge Books Online © Cambridge University Press, 2009

domains in signal transduction and the organisation of the actin cytoskeleton. The small, dysmorphic platelets in WAS-patients are due to a disorganized cytoskeleton.

A large number of mutations in *WASP* have been identified in patients with WAS[5], but also in patients with X-linked microthrombocytopenia (XLT). This disorder is not associated with eczema or increased susceptibility to infections and has a less severe clinical phenotype and prognosis. In these patients, the platelet count is moderately to severely reduced and bone marrow examination shows a normal presence of megakaryocytes. IgA is often increased with decreased isoagglutinins. The mutations in the *WASP* gene[6,7] in XLT are different from those found in classical WAS-patients. Why some mutations impair only the megakaryocytic lineage whereas others affect also the lymphoid cells is not yet clear. It seems however that, for predicting the clinical course, not only mutation analysis at the DNA level is important but also evaluation of the transcript at the mRNA level and at the protein level[8,9]: mutations in *WASP* leading to the absence of protein correlate with a more severe phenotype, whereas the translation of a normal-sized protein is more frequently found in patients with a milder clinical picture. Mutations affecting a splice site can be associated with either a severe or mild phenotype, depending on the proportion of normally spliced transcript generated and on the possible dominant negative effect of the abnormally spliced transcript on the normal protein.

Macrothrombocytopenia

X-linked macrothrombocytopenia has only recently been described and is associated with dyserythropoiesis[10]. In the princeps family the two affected half-brothers (same mother) had marked anemia due to dyserythropoiesis and an important bleeding problem related to severe thrombocytopenia (one was platelet-transfusion dependent and the other underwent bone marrow transplantation). The authors found in this family a missense mutation (V205M) in the N-terminal zinc-finger of GATA1. GATA1 is the founding member of the GATA-binding family of transcription factors and has been shown to be an essential protein for normal erythropoiesis and megakaryocyte differentiation[11,12]. The human gene encoding GATA1 has been mapped to Xp11.23[13]. The V205M mutation leads to a decreased binding of GATA1 to its essential cofactor FOG1 (for Friend Of GATA1). We recently found two families with other mutations in the same N-terminal zinc finger of GATA1. The first family with ten documented affected males has a missense mutation, resulting in the replacement of Asp by Gly at codon 218 (D218G), and a less severe

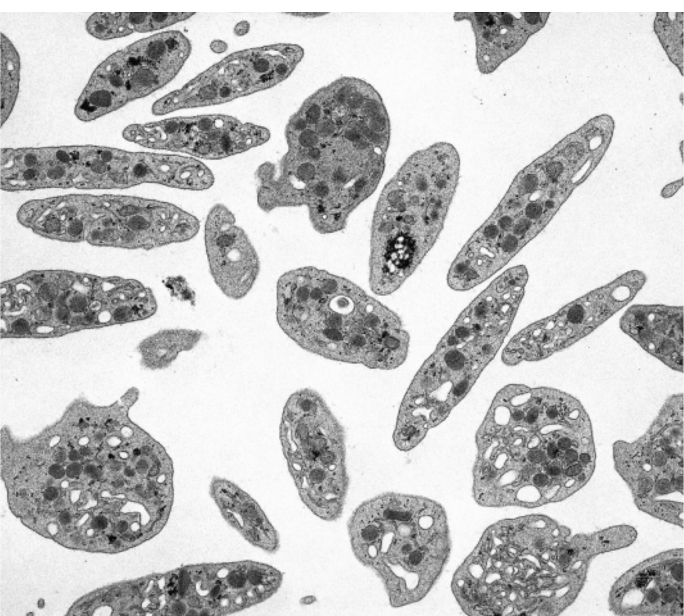


Fig. 34.1. Electron micrograph of normal human platelets. The platelets have a uniform appearance and show the typical discoid shape with normal organelles and platelet specific granules. (Original magnification: ×14 500.)

clinical picture than the previously published family; macrothrombocytopenia and dyserythropoiesis are moderate and with no marked anemia[14]. The second family has another missense mutation in the same codon with replacement of Asp by Tyr (D218Y). In this family the phenotype is very severe and even life threatening with only one affected member still alive; he has severe macrothrombocytopenia and pronounced dyserythropoietic anemia[15]. In each case, the interaction of the mutated GATA1 with FOG1 is disturbed: only mildly with the D218G mutant and markedly with the D218Y mutant, thereby correlating with the clinical phenotype. As compared to normal platelets (Fig. 34.1), the platelets in these GATA1-mutated patients have a characteristic ultrastructure (Fig. 34.2): in electron microscopy, these enlarged platelets are spherical rather than discoid, and contain clusters composed of smooth endoplasmic reticulum and abnormal membrane complexes both in the centre and in the periphery of their cytoplasma. Platelets of the hitherto described female carriers are normal in number and in ultrastructure. Light microscopically, the red blood cells are dysmorphic with anisocytes, poikilocytes, megalocytes and acanthocytes. The bone marrow of the affected patients with the D218G mutation shows normoblasts with punctate basophilia, karyorhexis, budding and pycnosis and also increased dysplastic megakaryocytes with megakaryoblasts,

Cambridge Books Online © Cambridge University Press, 2009

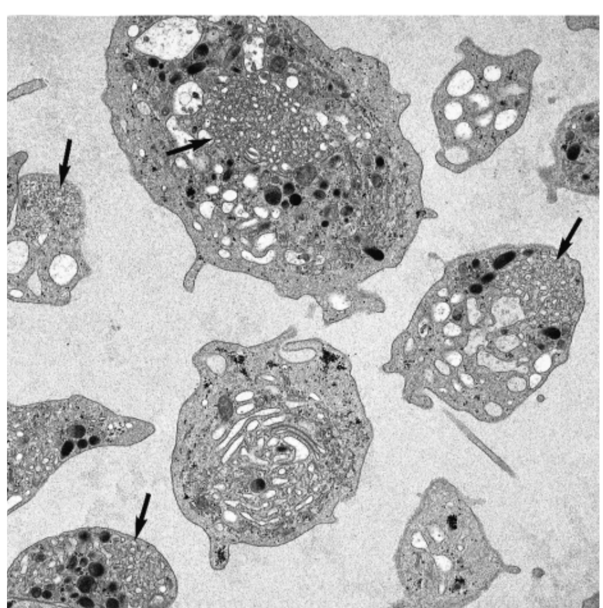


Fig. 34.2. Electron micrograph of platelets from a patient with inherited mutation in GATA-1. Enlarged platelets showing round inclusions (arrow) composed of smooth endoplasmic reticulum and abnormal membranous complexes localised in the centre or at the periphery of their cytoplasm. (Original magnification: × 14 500.)

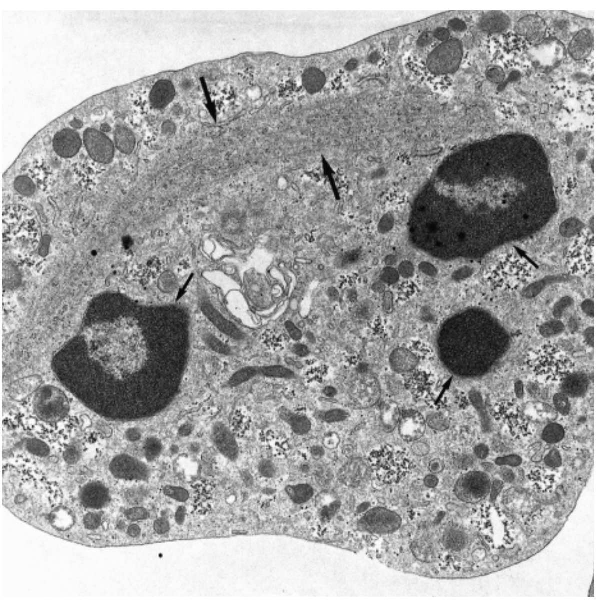


Fig. 34.3. Electron micrograph of a neutrophil from a patient with May–Hegglin anomaly. Spindle-shaped inclusions, containing a linear array of parallel filaments 7 to 10 nm diameter (arrow), orientated in the long axis of the inclusion. Part of lobulated nucleus (thin arrow). (Original magnification: × 23 000.)

micromegakaryocytes, vacuolization of the cytoplasma and asynchronous maturation of the nucleus and cytoplasma. The increased number of megakaryocytes in the bone marrow could potentially lead to the misdiagnosis of an acquired chronic ITP in these patients. The function of these platelets has been studied in the patients with the D218G mutation and shows a decreased but not absent ristocetin-induced agglutination due to a disturbed GPIb/V/IX complex. The promoters of many megakaryocyte-expressed genes have a GATA1 recognition site, as is the case for GPIbα, GPIbβ, GPIIb, GPIX, PF4, c-MPL and p45 NF-E2 and mutated GATA1 (D218G) platelets show a decreased GPIbβ and GPIX mRNA expression level.

The role of the GATA1–FOG1 transcription complex had already been demonstrated earlier in mice. Homozygous disruption of either GATA1 or FOG1 causes embryonic lethality due to a severe erythroid defect[12,16] and mice with a mutation in the upstream region of GATA1 (GATA1 knockout mice) show a milder erythroid defect but suffer from severe thrombocytopenia due to absent GATA1 expression in the megakaryocytic lineage[17]. The platelets in this latter mouse model[18] are enlarged and show a similar ultrastructure as in the above-mentioned patients. Therefore, it seems important that in patients with hereditary macrothrombocytopenia or so-called familial chronic idiopathic thrombocytopenic purpura, mutations in *GATA1* (and *FOG1*, once identified in humans) are looked for.

Autosomal dominant thrombocytopenia

Macrothrombocytopenia

May–Hegglin anomaly is an autosomal dominant disorder with high penetrance, first reported by May (1903)[19] and Hegglin (1945)[20] in which thrombocytopenia, giant platelets and basophilic Döhle-like inclusions are found in peripheral leukocytes (Fig. 34.3). These inclusions contain cytoplasmic RNA and are similar to those seen, although transiently, in acute infections[21].

In 1972, the Epstein syndrome was described as a variant of Alport syndrome in patients with renal disease, sensorineural deafness and macrothrombocytopenia and without leukocyte inclusion bodies[22]. Peterson presented a variant of the Epstein syndrome by describing a family with eight affected members in four generations with nephritis, deafness, congenital cataracts, macrothrombocytopenia, and also leukocyte inclusions. This new syndrome was called

Cambridge Books Online © Cambridge University Press, 2009

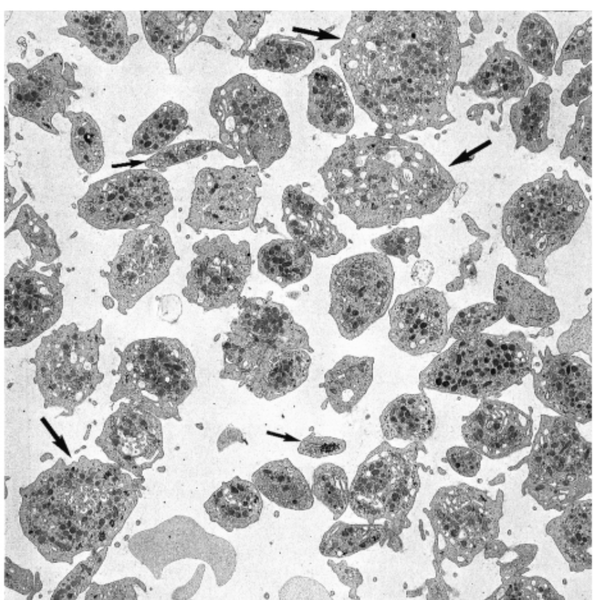


Fig. 34.4. Electron micrograph of platelets from a patient with Fechtner syndrome. Many round enlarged platelets are obvious (arrow). Some normal discoid platelets can be observed too (thin arrow). (Original magnification: ×5 750.)

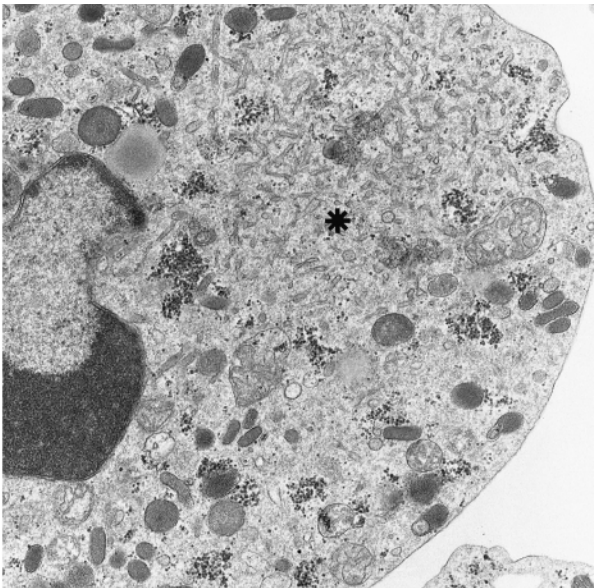


Fig. 34.5. Electron micrograph of neutrophil from a patient with Fechtner syndrome. Irregular not membrane-bound cytoplasmic inclusion is obvious (asterisk). Note granule-free zone containing numerous short cisternae of the smooth endoplasmic reticulum and some free dispersed ribosomes. (Original magnification: × 28 750.)

Fechtner syndrome, after the surname of this family[23] (Fig. 34.4). The leukocyte inclusions that are present in the neutrophils and eosinophils resemble light microscopically the Döhle bodies or the inclusions seen in May-Hegglin anomaly. However, ultrastructurally, these inclusions might be slightly different[24,25] (Fig 34.5). The renal disease varies from microscopic haematuria to end-stage renal failure. The Sebastian platelet syndrome has the same haematological characteristics (leukocyte inclusions and macrothrombocytopenia) as seen in Fechtner syndrome, but without the clinical expressions of the Alport syndrome[26]. Sebastian platelet syndrome and May– Hegglin anomaly therefore can only be differentiated by subtle ultrastructural leukocyte inclusion features: in both cases an oval cytoplasmic inclusion not bounded by membrane and lacking specific granules is present. In May–Hegglin anomaly (Fig. 34.3) the inclusion bodies contain clusters of ribosomes oriented along parallel filaments 7–10 nm in diameter whereas in Sebastian platelet syndrome (as in Fechtner syndrome, Fig. 34.5) they are composed of highly dispersed filaments and randomly distributed ribosomes[27]. There also was clinical evidence that Sebastian platelet syndrome is only one end of the clinical spectrum of Fechtner syndrome: a four generation family was diagnosed with characteristics of the Sebastian platelet syndrome (macrothrombocytopenia and leukocyte inclusions) and with silent ocular lens opacities as the only Alport-like manifestation[28]. Further evidence that these disorders are linked came from the genetic field. The disease loci for May–Hegglin anomaly and Fechtner syndrome both mapped to overlapping regions on chromosome 22. Also recently it became obvious that Sebastian platelet syndrome, May–Hegglin anomaly and Fechtner syndrome are allelic and have mutations in the same gene *MYH9*, encoding the 224 kD nonmuscle myosin heavy chain IIA (MYHIIA) polypeptide[27,29–31]. This MYHIIA is present in platelets, monocytes, granulocytes, the kidney and the auditory system but also in a lot of other tissues. Why the patients with May–Hegglin anomaly, Fechtner syndrome or Sebastian platelet syndrome do not have symptoms of the other affected tissues remains to be elucidated. The exact disease pathogenesis needs to be unravelled further as well as whether the Epstein syndrome could belong to the same family of disorders on a molecular level.

Grey platelet syndrome is a very rare inherited disorder of the α-granules of the platelets, first described by Raccuglia[32], and characterized by large agranular platelets (Fig. 34.6) and thrombocytopenia[33,34]. The platelets have a grey appearance when studied on Wright-stained blood smears due to the lack or paucity of α-granules, with,

Cambridge Books Online © Cambridge University Press, 2009

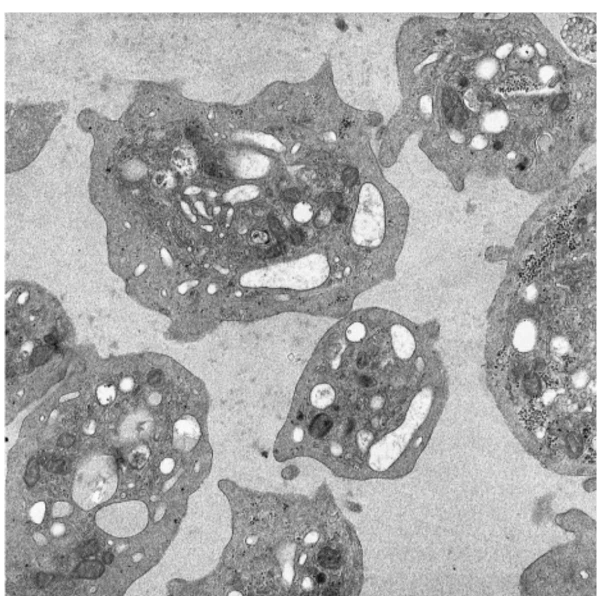


Fig. 34.6. Electron micrograph of platelets from a patient with grey platelet syndrome. Enlarged platelets showing almost complete lack of normal alpha granules. Note a marked vacuolization of their cytoplasm and dilated cisternae of the surface connected canalicular system (s.c.c.s.). (Original magnification: $\times$23 000.)

however, a normal content of dense granules. Most cases are sporadic, but the familial pedigree studies suggest an autosomal dominant inheritance. The range of thrombocytopenia is very broad (from 20×10^9/l to normal) and even patients with normal platelet count have a bleeding problem due to the qualitative defect of their platelets (see also chapter on platelet release defect disorder). In this syndrome there is a normal synthesis but a defective packaging of the soluble proteins normally present in these granules: von Willebrand factor (VWF), fibrinogen, thrombospondin, platelet-derived growth factor (PDGF), albumin, fibronectin, thrombospondin, platelet factor 4, β-thromboglobulin, plasminogen activator inhibitor I (PAI1) and factor V. The α-granule membrane proteins, P-selectin, GP IIbIIIa, CD36, and GPIb/V/IX, are expressed normally. The hemostasis in these patients can be improved by platelet transfusion, inhibitors of fibrinolysis or even DDAVP, thereby probably releasing VWF from other cellular stores. The molecular pathogenesis of this syndrome remains unknown.

Von Willebrand Disease (VWD) type 2B is an autosomal dominant bleeding disorder in which large but qualitatively abnormal von Willebrand factor (VWF) multimers are synthesized, that spontaneously bind to the GPIbα on the platelets. As a result of this, patients show a decreased presence of these large multimers in the plasma and a decreased ristocetin cofactor activity. Platelets, moreover, agglutinate at low concentrations of ristocetin that have no effect on normal platelets. Patients with type 2B VWD often demonstrate thrombocytopenia that is variable in time and more pronounced in conditions where the VWF is increased, such as pregnancy or following DDAVP infusion. Nurden et al.[35] demonstrated that the platelets of patients with type 2B VWD have ultrastructural abnormalities. The platelets are large and spherical with zones of membrane complexes and a dilated surface-connecting system containing amorphous material. There are huge amounts of VWF colocalized with GPIb on the surface and within the platelet surface connected canalicular system.

The molecular defects in type 2B VWD are missense mutations located in exon 28 of the gene encoding VWF, leading to amino acid alterations in the disulphide loop between Cys^{509} and Cys^{695}[36]. Therefore, in the differential diagnosis of autosomal dominant macrothrombocytopenia, this type of VWD has to be taken into account.

Platelet-type or pseudo- von Willebrand disease has the same clinical features as the above-mentioned type 2B VWD, but the increased affinity of VWF for the GPIb complex in this condition is due to a mutation in the GPIbα gene instead of in the VWF gene. The platelet number is normal to decreased and the mean platelet volume is mostly increased[37]. The two described mutations in patients with this bleeding disorder, G233V[38] and M239V[39], enrich the valine content of an already valine-rich region within the Cys^{209}–Cys^{248} disulfide loop of GPIbα and thereby are gain-of-function mutations.

Patients with 22q11 microdeletion have mild thrombocytopenia and giant platelets. The clinical picture of this syndrome is very broad (Fig. 34.7): it ranges from the more severe DiGeorge sequence (thymic hypoplasia, conotruncal cardiac defects, and facial dysmorphy) to the milder velocardiofacial syndrome (VCFS) with velopharyngeal insufficiency, conotruncal heart disease and learning disabilities. Levy et al.[40] first reported two female patients with del 22q11 and thrombocytopenia, although the nature of this thrombocytopenia was not clear. Budarf et al.[41] described a boy with del 22q11 and Bernard–Soulier syndrome (BSS). He was a compound heterozygote with the deletion of one allele of the GPIbβ gene, that maps to the deleted 22q11.2 region, and a mutated GATA binding site in the promoter of the remaining allele[42]. Knowing that patients with del 22q11 are obligate carriers of a GPIbβ deletion, these patients can be considered as heterozygous BSS. The platelet count ranges from mildly reduced to normal, and the platelets have an increased size (Fig. 34.8).

Cambridge Books Online © Cambridge University Press, 2009

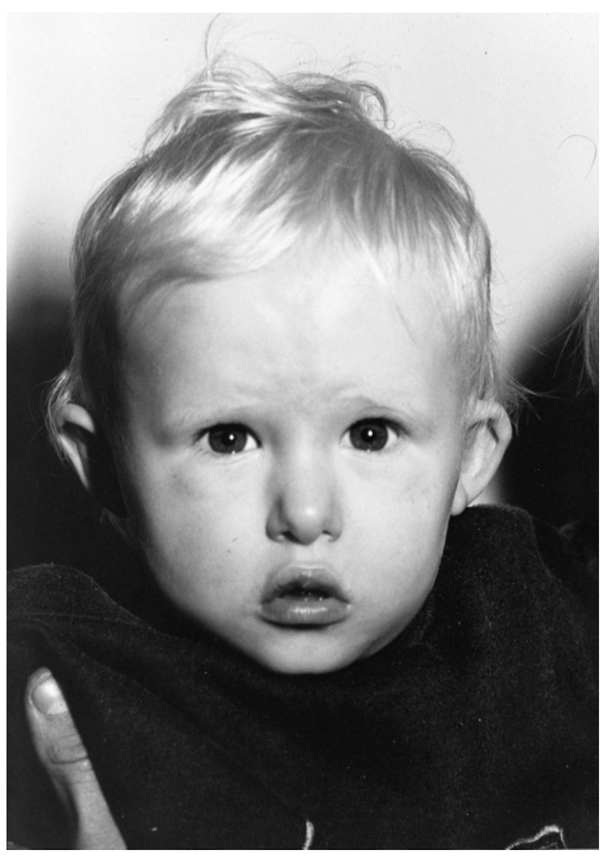


Fig. 34.7. Clinical picture of a patient with 22q11 microdeletion. Note the typical prominant nose with squared nasal root and narrow alar base and auricular abnormalities.

The platelet function however, as tested by platelet aggregation studies and by platelet adhesion studies to collagen in a whole blood perfusion system, is normal[43]. Therefore these patients have only a mild bleeding tendency (if any) that correlates directly with the decrease in platelet count.

Montreal platelet syndrome[34] is an autosomal dominant disorder characterised by giant platelets in the peripheral blood, thrombocytopenia and spontaneous platelet aggregation, probably contributing to the pathophysiology of the thrombocytopenia. The platelet membrane glycoproteins are normal but there seems to be a defect in calpain (calcium-activated neutral proteinase)[44]. The molecular mechanism underlying this disorder is not unravelled yet.

Thrombocytopenia with normal platelet size

Autosomal dominant thrombocytopenia with normal platelet size was first described in 11 members of a four-generation family[45]. Thereafter several other families have

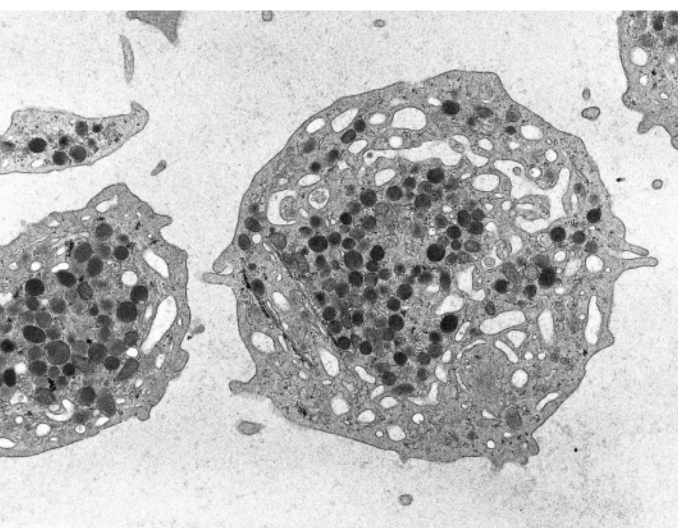


Fig. 34.8. Electron micrograph of a giant round platelet from a patient with 22q11 microdeletion. Numerous alpha granules, rather segregated in the centre, can be seen. (Original magnification: $\times$14 500.)

been reported with mild to moderate thrombocytopenia inherited in a dominant way. Iolascon et al.[46] described a large Italian family with moderate thrombocytopenia in 17 affected members, normal platelet size, normocellular bone marrow and normal platelet function. In the affected patients, the TPO-levels were elevated and the response to TPO was normal, making a defect in the TPO-production or in the TPO-receptor (c-mpl) very unlikely. Moreover, linkage studies to chromosome 3q26.3–q27 and 1p34, regions containing the TPO and c-mpl genes, respectively, could completely rule out this pathogenicity. Other candidate loci on 11q23–24 and 21q11 were excluded. Savoia et al.[47] performed a genomewide search in the same family and demonstrated by microsatellite analysis that the disease locus is linked to chromosome 10p11,1–12 with a candidate region of 6 cM. Recently 13 affected members of another family were studied[48] with a mean platelet count of 42.7×10^9/l (range 18×10^9/l to 106x 10^9/l); males and females were equally affected, compatible with an autosomal dominant mode of inheritance. These patients had no major bleeding diathesis, suggesting normal function of the remaining platelets. Megakaryocytes in the bone marrow are reduced, with delayed nuclear and cytoplasmic maturation. There are no obvious abnormalities in the myeloid or the erythroid cells. Affected members had modestly increased thrombopoietin levels. The medical history of some of these patients revealed no effect of immunosuppression or splenectomy on the platelet count. Genome-wide linkage studies based on 30 members of this

Cambridge Books Online © Cambridge University Press, 2009

family have again identified a 17-cM region on chromosome 10p. Further studies are presently looking for the responsible gene in this region.

Paris-Trousseau thrombocytopenia is a dysmegakaryopoietic disorder due to a cytogenetic abnormality. Wardinsky et al.[49] reported 35 patients with deletion of 11q23 associated with mental retardation, trigonocephaly, depressed nasal bridge, hypertelorism, and cardiac and neurological malformations, of whom 47% suffered from thrombocytopenia. Favier[50] described a mother and son with del 11q23, mental retardation, dysmorphic features such as hypertelorism, long philtrum and low set ears, and chronic thombocytopenia with mild bleeding complications. This condition is characterized by giant α-granules in 15% of platelets in the peripheral blood, resulting from fusion of the α-granules. These giant α-granules do not release their content after stimulation, in contrast to platelets with normal morphology. Platelet lifespan is normal but, in the bone marrow, an increased number of micromegakaryocytes is found with premature megakaryocytic apoptosis evidenced by massive cell lysis at the end of maturation[51]. Probably the Paris–Trousseau thrombocytopenia can be seen as a contiguous gene syndrome as the proto-oncogenes ETS1 and FLI1 map to 11q23.3 and 11q24, respectively, and are absent in terminal 11q23 deletion. They are both essential, along with GATA-1, for the normal expression of megakaryocyte-specific genes[52].

Factor V Quebec was first described by Tracy[53] and is characterized by dominantly inherited mild thrombocytopenia and moderate bleeding problems. The platelet factor V activity is less than 6% of normal and platelet factor V antigen levels are half of normal. This decreased factor V is accompanied by a defect in multimerin[54]. Multimerin is a large, complex, multimeric protein and stabilizes factor V within the platelet α-granule. These patients furthermore have a decreased content in their α-granules not only of factor V but also of fibrinogen, VWF, thrombospondin and osteonectin, possibly by increased degradation of the protein content[55]. The normal content of platelet factor 4 and β-thromboglobulin suggests a normal assembly of the α-granules. The platelets demonstrate a functional defect in their epinephrine-mediated aggregation[56]. The explanation why factor V Quebec leads to thrombocytopenia is currently unknown, neither is the genetic mechanism unravelled.

Familial platelet disorder with predisposition to acute myelogenous leukemia (FPD/AML) is an autosomal dominant disorder characterised by qualitative and quantitative platelet defects and in which the affected members have an increased risk of developing AML[57]. The thrombocytopenia in the first described family, with 22 family members definitively affected, ranged from 30×10^9/l to 150×10^9/l without qualitative or quantitative abnormalities of red or white blood cells. Moreover, a thrombopathy was obvious with decreased platelet aggregation after stimulation with collagen and epinephrine, contributing to the defective haemostasis. Electron microscopy of the platelets revealed normal platelet size and presence of all organelles in adequate quantity and configuration. The platelet survival was normal in these patients. Six of them developed hematologic neoplasms. Ho[58] performed linkage analysis on this originally described family, and found a critical region of 15.2 cM on chromosome 21q22.1–22.2. Finally, by mutational analysis of the candidate genes in the region where FPD/AML maps, a heterozygous mutation of the hematopoietic transcription factor *CBFA2* (formerly *AML1*) was discovered leading to a decrement of megakaryocyte colony formation[59]. It was also suggested that the haploinsufficiency of *CBFA2* may predispose to acquisition of second mutations at other loci that are leukemogenic.

Autosomal recessive thrombocytopenia

Macrothrombocytopenia

Bernard–Soulier syndrome (BSS) is an autosomal recessive bleeding disorder with unusually large platelets and a moderate degree of thrombocytopenia, first described by Bernard and Soulier[60]. These platelets have an important functional defect: they aggregate normally after stimulation by ADP, collagen or arachidonic acid but they do not agglutinate with ristocetin[61] (see also chapter on Platelet adhesive protein defect disorders). This is due to a defective GPIb/V/IX complex, the major receptor for von Willebrand factor[62] with also binding properties for thrombin. As GPIb is the major sialylated glycoprotein on the platelet surface, and as asialylated platelets have a shortened survival[63], absence of this receptor can give rise to thrombocytopenia. BSS has mostly been related to mutations in the gene for GP Ib-α (BSS type A) but also in the gene for GPIb-β (BSS type B) and in the gene for GPIX (BSS type C). Ware et al.[64] disrupted the GPIbα-gene in mice; this resulted in the BSS phenotype. Moreover, he rescued the BSS phenotype by expressing the human GP Ibα subunit on the surface of the circulating platelets. No BSS patient with a mutation in the GPV gene has been found. This is consistent with the finding that GPV knockout mice have normal megakaryocytes with normal GPIb–IX expression on the cell surface[65].

Cambridge Books Online © Cambridge University Press, 2009

Thrombocytopenia with normal platelet size

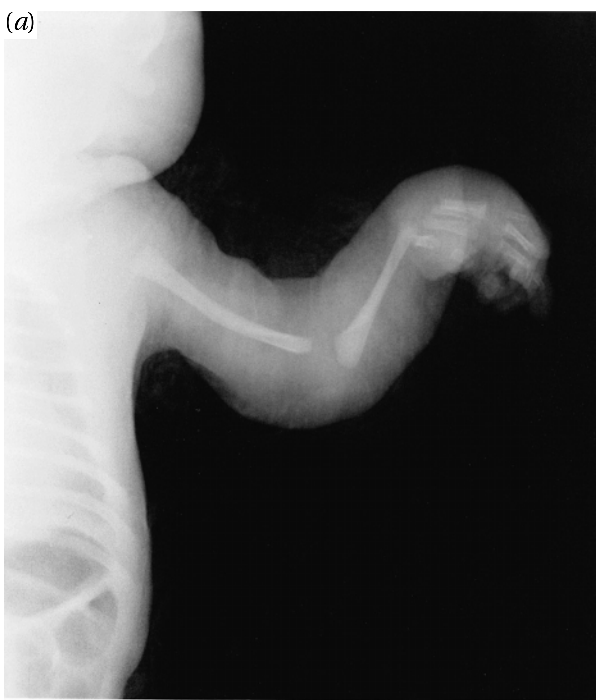
Congenital amegakaryocytic thrombocytopenia (CAMT) without associated physical anomalies is a rare disorder, characterised by isolated thrombocytopenia and megakaryocytopenia in infancy. It is considered a constitutional marrow failure syndrome, because of evolution with time towards bone marrow aplasia and leukemia[66]. The pathogenesis of this disorder remained unsolved until recently, with increasing insight into the mechanisms of megakaryocyte proliferation and differentiation. In 1994, several groups purified the protein thrombopoietin (TPO) or cloned the cDNA for TPO[67]. It became clear that TPO is the principal regulator of megakaryocytic cell proliferation and differentiation. The TPO-receptor gene (c-mpl) belongs to the cytokine receptor superfamily and encodes for five coexpressed mRNA isoforms. It is expressed in the megakaryocytic lineage from late progenitors to platelets[68]. TPO cannot be replaced by other cytokines in many functions: the formation of platelet specific granules, the development of demarcation membranes and platelet fields, the expression of platelet-specific membrane proteins (GP IIb/IIIa and GP Ib/V/IX), megakaryocyte adhesion through activation of GP IIb/IIIa, VLA-4, and VLA-5, endomitosis and its resultant polyploid state and finally the formation of platelets from single megakaryocytes in serum-free cultures[69]. Moreover, TPO and c-mpl also play a role in early hematopoiesis by supporting the survival of early hematopoietic progenitors and by inducing the proliferation of megakaryocytic progenitor cells by synergistic action with interleukin (Il)-3, stem-cell factor, Il-11, and EPO. The mechanism by which TPO exerts this action is probably by decreasing progenitor cell apoptosis[70].

TPO plasma levels are elevated in patients with CAMT[71,72], in line with the finding that TPO levels are high when the thrombocytopenia is due to megakaryocyte deficiency and low when it is due to peripheral destruction[73]. Furthermore, a CAMT patient had an in vitro defective response to TPO in megakaryocyte-colony formation, with decreased numbers of erythroid and myelocytic progenitors in clonal cultures and a lack of c-mpl mRNA in bone-marrow mononuclear cells[71]. Further evidence that c-mpl might be the candidate gene for CAMT without associated physical anomalies came from animal studies. In mice a deficiency of the c-mpl gene results in amegakaryocytic thrombocytopenia, decreased numbers of progenitors and increased concentrations of circulating TPO without any other clinical abnormalities[74–77]. Due to the similarity between human CAMT and murine c-mpl deficiency, this gene was studied in patients with CAMT and a compound heterozygote with two truncating mutations in c-mpl was identified[78] as well as a compound heterozygote for two missense mutations[72]. Van den Ouderijn et al.[79] found mutations in the c-MPL gene in 4 out of 5 CAMT patients, confirming that CAMT is an autosomal recessive disorder that can be due to c-mpl mutations.

Congenital thrombocytopenia with absent radius is referred to by the acronym TAR-syndrome and is characterized by hypomegakaryocytic thrombocytopenia ($<100 \times 10^9$/l) and bilateral absence of the radius with normal thumbs (Fig. 34.9). Initially, this condition was considered to be a form of Fanconi's anemia. However, the natural history of TAR-syndrome is more benign as compared to Fanconi's anemia. The majority of affected families suggested an autosomal recessive inheritance, whereas in some families the TAR-syndrome seemed to be inherited in a autosomal dominant way. Hall reviewed the phenotypic features of this syndrome[80]. 100% of the TAR-patients demonstrate a thrombocytopenia, which is symptomatic in 90% of the patients during the first months of life. Mostly, the platelet count in infancy ranges between 15×10^9/l and 30×10^9/l and thereafter there is a gradual improvement towards almost normal levels by adulthood. Bone marrow shows small, basophilic and vacuolated megakaryocytes. Leukemoid reactions are seen during the first year of life in two-thirds of the patients. True leukemia has never been documented in a patient with TAR-syndrome. The often observed anemia could either be a consequence of the blood loss, due to the thrombocytopenia, or due to a shortened RBC lifespan. The bilateral absence of the radius with the presence of normal thumbs is obligatory for the diagnosis; however, numerous additional skeletal abnormalities have been described, such as abnormal ulna, humerus, or shoulder; one-half of the TAR-patients also have abnormalities of the legs. During embryonic development, disturbances in the heart formation can occur with tetralogy of Fallot and atrial septal defects as most common problems. There is a marked inter- and even intrafamilial variability as to the extent of the skeletal, hematological and cardiac anomalies. The final vital prognosis for an affected child with TAR-syndrome is normal if the child survives the first few years of life.

The molecular defect leading to this syndrome is currently unknown. Elevated serum levels of thrombopoietin (TPO) have been found in patients with TAR-syndrome. The reactivity of TAR-platelets in vitro towards recombinant TPO was decreased, despite normal expression of the TPO-receptor on the surface of the platelets[81]. Strippoli et al.[82] performed mutational screening of the promoter of the TPO-receptor gene (c-mpl) and the coding region of the c-mpl gene in four unrelated TAR-patients and found no mutations thereby excluding c-mpl mutations as a

Cambridge Books Online © Cambridge University Press, 2009



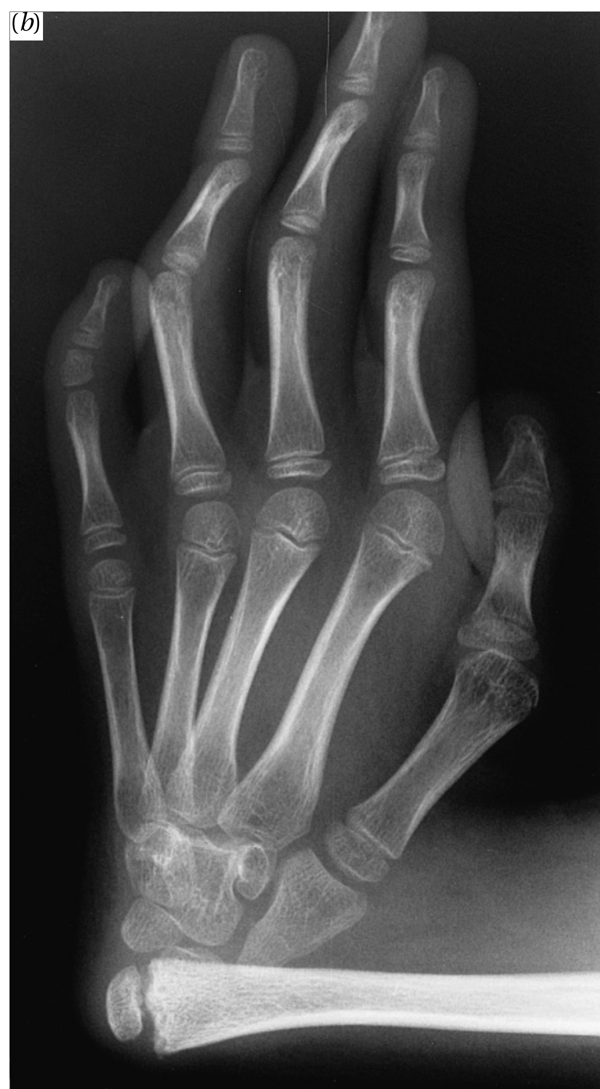


Fig. 34.9. X-ray photographs of the upper limb of a patient with TAR-syndrome. Note the absence of the radius at infancy (*a*) and at the age of 10 years (*b*) with normal hand development.

common cause of thrombocytopenia in TAR-syndrome. Further evaluation of the signal transduction following TPO-c-mpl interaction could possibly clarify the pathogenesis of this syndrome.

Conclusions

Although rare, the hereditary thrombocytopenias allow to identify a number of proteins clearly involved in human thrombopoiesis and will help to clarify further this complex biological phenomenon. What is striking from the overview, is that different mutations in the same gene, or even within the same codon of the gene, can lead to markedly dissimilar phenotypes: Wiskott–Aldrich syndrome or X-linked microthrombocytopenia, the different severities in X-linked macrothrombocytopenia due to GATA-1 mutations, the May–Hegglin anomaly, Sebastian platelet syndrome or the Fechtner syndrome. On the other hand, mutations in two different genes, but with interactions between their gene products, can result in very similar phenotypes: von Willebrand disease type 2B and the platelet-type von Willebrand disease. Careful dissection of the experiments of nature resulting in hereditary disorders continues to be a major source of medical insight.

References

1 Buckley RH. Primary immunodeficiency diseases due to defects in lymphocytes. *N Engl J Med* 2000; 343; 1313–1324.
2 Mullen CA, Anderson KD, Blaese RM. Splenectomy and/or bone marrow transplantation in the management of the Wiskott–Aldrich syndrome: long-term follow-up of 62 cases. *Blood* 1993; 82: 2961–2966.
3 Derry JMJ, Ochs HD, Francke U. Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. *Cell* 1994; 78: 635–644.
4 Featherstone C. The many faces of WAS protein. *Science* 1997; 275: 27–28.

Cambridge Books Online © Cambridge University Press, 2009

5 Schwarz K. WASPbase: a database of Was- and XLT-causing mutations. *Immunol Today* 1996; 17: 496–502.
6 Villa A, Notarangelo L, Macchi P et al. X-linked thrombocytopenia and Wiskott–Aldrich syndrome are allelic diseases with mutations in the Wasp gene. *Nat Genet* 1995; 9: 414–417.
7 Zhu Q, Zhang M, Blaese RM et al. The Wiskott–Aldrich syndrome and X-linked congenital thrombocytopenia are caused by mutations of the same gene. *Blood* 1995; 86: 3797–3804.
8 Ochs H. The Wiskott–Aldrich syndrome. *Semin Hematol* 1998; 35: 332–345.
9 Lemahieu V, Gastier JM, Francke U. Novel mutations in the Wiskott–Aldrich syndrome protein gene and their effects on transcriptional, translational, and clinical phenotypes. *Hum Mutat* 1999; 14: 54–66.
10 Nichols KE, Crispino JD, Poncz M et al. Familial dyserythropoietic anaemia and thrombocytopenia due to an inherited mutation in GATA-1. *Nat Genet* 2000; 24: 266–270.
11 Tsai SF, Martin DI, Zon LI, Danfdrea AD, Wong GG, Orkin SH. Cloning of cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells. *Nature* 1989; 339: 446–451.
12 Pevny L, Simon MC, Robertson E et al. Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* 1991; 349: 257–260.
13 Zon LI, Tsai SF, Burgess S, Matsudaira P, Bruns GAP, Orkin SH. The major human erythroid DNA-binding protein (GF-1): primary sequence and localization of the gene to the X chromosome. *Proc Natl Acad Sci* 1990; 87: 668–672.
14 Freson K, Devriendt K, Matthijs G et al. Platelet characteristics in patients with X-linked macrothrombocytopenia because of a novel GATA1 mutation. *Blood* 2001; 98: 85–92.
15 Freson K, Matthijs G, Thys C et al. Different substitutions at residue D218 of the X-linked transcription factor GATA1 lead to altered clinical severity of macrothrombocytopenia and anemia and are associated with variable skewed X inactivation. *Hum Molec Genet*, in press.
16 Tsang AP, Fujwara Y, Hom DB, Orkin SH. Failure of megakaryopoiesis and arrested erythropoiesis in mice lacking the GATA1 transcriptional cofactor FOG. *Genes Dev* 1998; 12: 1176–1188.
17 Shivdasani RA, Fujiwara Y, McDevitt MA, Orkin SH. A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. *EMBO J* 1997; 16: 3965–3973.
18 Vyas P, Ault K, Jackson CW, Orkin SH, Shivdasani RA. Consequences of GATA-1 deficiency in megakaryocytes and platelets. *Blood* 1999; 93: 2867–2875.
19 May R. Leukozyteneinschlusse. *Dtch Arch Klin Med* 1909; 96: 1–6.
20 Hegglin R. Gleichzeitige konstitutionelle Veranderungen an Neutrophilen und Thrombozyten. *Helv Med Acta* 1945; 12: 439–440.
21 Jenis EH, Takeuchi A, Dillon DE, Ruymann FB, Rivkin S. The May–Hegglin anomaly: ultrastructure of the granulocyte inclusion. *Am J Clin Path* 1971; 55: 187–196.
22 Epstein CJ, Sahud MA, Piel CF et al. Hereditary macrothrombocytopenia, nephritis and deafness. *Am J Med* 1972; 52: 299–310.
23 Peterson LC, Rao KV, Crosson JT, White JG. Fechtner syndrome: a variant of Alport's syndrome with leukocyte inclusions and macrothrombocytopenia. *Blood* 1985; 65: 397–406.
24 Heynen MJ, Blockmans D, Verwilghen RL, Vermylen J. Congenital macrothrombocytopenia, leukocyte inclusions, deafness, and proteinuria: functional and electron microscopic observations on platelets and megakaryocytes. *Br J Haematol* 1988; 70: 441–448.
25 Greinacher A, Mueller-Eckhardt C. Hereditary types of thrombocytopenia with giant platelets and inclusion bodies in the leukocytes. *Blut* 1990; 60: 53–60.
26 Greinacher A, Nieuwenhuis HK, White JG. Sebastian platelet syndrome: a new variant of hereditary macrothrombocytopenia with leukocyte inclusions. *Blut* 1990; 61: 282–288.
27 May-Hegglin/Fechtner Consortium. Mutations in MYH9 result in the May-Hegglin anomaly, and Fechtner and Sebastian syndromes. *Nat Genet* 2000; 26: 103–105.
28 Rocca B, Laghi F, Zini G, Maggiano N, Landolfi R. Fechtner syndrome: report of a third family and literature review. *Br J Haematol* 1993; 85: 423–426.
29 Kelley MJ, Jawien W, Lin A et al. Autosomal dominant macrothrombocytopenia with leukocyte inclusions (May–Hegglin anomaly) is linked to chromosome 22q12–13. *Hum Genet* 2000; 106: 557–564.
30 Kelley MJ, Jawien W, Ortel TL, Korzack JF. Mutation of MYH9, encoding non-muscle myosin heavy chain A, in May–Hegglin anomaly. *Nat Genet* 2000; 26: 106–108.
31 Toren A, Rozenfeld-Granot G, Rocca B et al. Autosomal-dominant giant platelet syndromes: a hint of the same genetic defect as in Fechtner syndrome owing to a similar genetic linkage to chromosome 22q11–13. *Blood* 2000; 96: 3447–3451.
32 Raccuglia G. Gray platelet syndrome: a variety of qualitative platelet disorders. *Am J Med* 1971; 51: 818–828.
33 Smith MP, Cramer EM, Savidge GF. Megakaryocytes and platelets in α-granule disorders. *Baillière's Clin Haematol* 1997; 10: 125–148.
34 Bellucci S. Megakaryocytes and inherited thrombocytopenias. *Baillière's Clin Haematol* 1997; 10: 149–162.
35 Nurden P, Chretien F, Poujol C, Winckler J, Borel-Derlon A, Nurden A. Platelet ultrastructural abnormalities in three patients with type 2B von Willebrand disease. *Br J Haematol* 2000; 110: 704–714.
36 Sadler JE. Biochemistry and genetics of von Willebrand factor. *Ann Rev Biochem* 1998; 67: 395–424.
37 Takahashi H, Handa M, Watanabe K et al. Further characterization of platelet -type von Willebrand's disease in Japan. *Blood* 1984; 64: 1254–1262.
38 Miller JL, Cunningham D, Lyle VA, Finch CN. Mutation in the gene encoding the α-chain of platelet glycoprotein Ib in platelet-type von Willebrand disease. *Proc Natl Acad Sci USA* 1991; 88: 4761–4765.

Cambridge Books Online © Cambridge University Press, 2009

39 Russell SD, Roth GJ. Pseudo-von Willebrand disease: a mutation in the platelet glycoprotein Ibα gene associated with a hyperactive surface receptor. *Blood* 1993; 81: 1787–1791.
40 Lévy A, Michel G, Lemerrer M, Philip N. Idiopathic thrombocytopenic purpura in two mothers of children with DiGeorge sequence: a new component manifestation of Catch22. *Am J Med Genet* 1997; 69: 356–359.
41 Budarf ML, Konkie BA, Ludlow LB et al. Identification of a patient with Bernard-Soulier syndrome and a deletion in the DiGeorge/velo-cardio-facial chromosomal region in 22q11.2. *Hum Mol Gen* 1995; 4: 763–766.
42 Ludlow LB, Schick BP, Budarf ML et al. Identification of a mutation in a GATA binding site of the platelet glycoprotein Ibβ promotor resulting in the Bernard–Soulier syndrome. *J Biol Chem* 1996; 271: 22076–22080.
43 Van Geet C, Devriendt K, Eyskens B, Vermylen J, Hoylaerts MF. Velocardiofacial syndrome patients with a heterozygous chromosome 22q11 deletion have giant platelets. *Pediatr Res* 1998; 44: 607–611.
44 Okita JR, Frojmovic MM, Kristopeit S et al. Montreal platelet syndrome: a defect in calcium activated neutral proteinase (calpain). *Blood* 1989; 74: 715–721.
45 Bithell TC, Didisheim P, Cartwright GE, Wintrobe MM. Thrombocytopenia inherited as an autosomal dominant trait. *Blood* 1965; 25: 231–240.
46 Iolascon A, Perrotta S, Amendola G et al. Familial dominant thrombocytopenia: clinical, biological, and molecular studies. *Pediatr Res* 1999; 46: 548–565.
47 Savoia A, Del Vecchio ME, Totaro A et al. An autosomal dominant thrombocytopenia gene maps to chromosomal region 10p. *Am J Hum Genet* 1999; 65: 1401–1405.
48 Drachman JG, Jarvik GP, Mehaffey MG. Autosomal dominant thrombocytopenia: incomplete megakaryocyte differentiation and linkage to human chromosome 10. *Blood* 2000; 96: 118–125.
49 Wardinsky TD, Weinberger ED, Pagon RA, Sterling KC, Thuline HC. Partial deletion of the long arm of chromosome 11 (11q23.3-qter) with abnormal white matter. *Am J Med Genet* 1990; 35: 60–63.
50 Favier R, Douay L, Esteva B et al. A novel genetic thrombocytopenia (Paris–Trousseau) associated with platelet inclusions, dysmegakaryopoiesis and chromosome deletion at 11q23. *CR Acad Sci* 1993; 316: 698–701.
51 Breton-Gorius J, Favier R, Guichard J et al. A new congenital dysmegakaryopoietic thrombocytopenia (Paris-Trousseau) associated with giant platelet α-granules and chromosome 11 deletion at 11q23. *Blood* 1995; 85: 1805–1814.
52 Lemarchandal V, Ghysdael J, Mignotte V et al. Gata and its cis-acting sequences mediate megakaryocyte-specific expression. *Mol Cell Biol* 1993; 13: 668–676.
53 Tracy PB, Giles AR, Mann KG et al. Factor V (Quebec): a bleeding diathesis associated with a qualitative platelet factor V deficiency. *J Clin Invest* 1984; 74: 1221–1228.
54 Hayward CPM, Furmaniak-Kazmierczak E, Cieutat AM et al. Factor V is complexed with multimerin in resting platelet lysates and colocalizes with multimerin within platelet alpha granules. *J Biol Chem* 1995; 270: 19217–19224.
55 Janeway CM, Rivard GE, Tracy PB, Mann KG. Factor V Quebec revisited. *Blood* 1996; 87: 3571–3578.
56 Hayward CPM, Rivard GE, Kane WH et al. An autosomal dominant, qualitative platelet disorder associated with multimerin deficiency, abnormalities in platelet factor V, thrombospondin, von Willebrand factor, and fibrinogen and an epinephrine aggregation defect. *Blood* 1996; 87: 4967–4978.
57 Dowton SB, Beardsley D, Jamison D, Blattner S, Li FP. Studies of a familial platelet disorder. *Blood* 1985; 65: 557–563.
58 Ho CY, Otterud B, Legare RD et al. Linkage of a familial platelet disorder with a propensity to develop myeloid malignancies to human chromosome 21q22.1–22.2. *Blood* 1996; 87: 5218–5224.
59 Song WJ, Sullivan MG, Legare RD et al. Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia. *Nat Genet* 1999; 23: 166–174.
60 Bernard J, Soulier J-P. Sur une nouvelle variété de dystrophie thrombocytaire-hémoragipare congénitale. *Semin Hop Paris* 1948; 24: 3217–3223.
61 Caen J, Levy-Toledano S. Interaction between platelets and von Willebrand factor provides a new scheme for primary haemostasis. *Nature New Biol* 1973; 244: 159–160.
62 Nurden AT, Caen JP. Specific roles for platelet surface glycoproteins in platelet function. *Nature* 1975; 225: 720–722.
63 Gröttum KA, Solum NO. Congenital thrombocytopenia with giant platelets: a defect in the platelet membrane. *Br J Haematol* 1969; 16: 277–290.
64 Ware J, Russell S, Ruggeri ZM. Generation and rescue of a murine model of platelet dysfunction: the Bernard-Soulier syndrome. *Proc Natl Acad Sci USA* 2000; 97: 2803–2808.
65 Poujol C, Ramakrishnan V, Deguzman F, Nurden AT, Philips DR, Nurden P. Ultrastructural analysis of megakaryocytes in GPV knockout mice. *Thomb Haemostas* 2000; 84: 312–318.
66 Alte BP, Young SY. Amegakaryocytic thrombocytopenia. In: Nathan DG, Oski FA eds. *Hematology of Infancy and Childhood*, 5th edn, Philadelphia: WB Saunders, 1998; 1: 281–282.
67 Kaushansky K. Thrombopoietin: the primary regulator of platelet production. *Blood* 1995; 86: 419–431.
68 Debili N, Wendling F, Cosman D et al. The Mpl receptor is expressed in the megakaryocytic lineage from late progenitors to platelets. *Blood* 1995; 85: 391–401.
69 Kaushansky K. Thrombopoietin: from theory to reality. *Thromb Haemost* 1999; 82: 312–317.
70 Nagasawa T, Osada M, Komeno T et al. In vitro and in vivo deprivation of thrombopoietin induces megakaryocytic apoptosis and regulates platelet production. *Exp Hematol* 1997; 25: 897.
71 Muraoka K, Ishii E, Tsuji K, Yamamoto S, Yamaguchi H, Hara T, Koga H. Defective response to thrombopoietin and impaired expression of c-mpl mRNA of bone marrow cells in congenital amegakaryocytic thrombocytopenia. *Br J Haematol* 1997; 96: 287–292.
72 Tonelli R, Scardovi AL, Pession A et al. Compound heterozy-

Cambridge Books Online © Cambridge University Press, 2009

gosity for two different amino-acid substitutions in the thrombopoietin receptor (c-mpl gene) in congenital amegakaryocytic thrombocytopenia (CAMT). *Hum Genet* 2000; 107: 225–233.

73 Emmons RV, Reid DM, Cohen RL et al. Human thrombopoietin levels are high when thrombocytopenia is due to megakaryocyte deficiency and low when due to increased platelet destruction. *Blood* 1996; 87: 4068–4071.

74 Alexander WS, Roberts AW, Nicola NA, Li R, Metcalf D. Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl. *Blood* 1996; 87: 2162–2170.

75 Carver-Moore K, Broxmeyer HE, Luoh SM et al. Low levels of erythroid and myeloid progenitors in thrombopoietin- and c-mpl-deficient mice. *Blood* 1996; 88: 803–808.

76 Gurney AL, Carver-Moore K, Sauvage FJ de, Moore MW. Thrombocytopenia in c-mpl deficient mice. *Science* 1994; 265: 1445–1447.

77 Kimura S, Roberts AW, Metcalf D, Alexander WS. Hematopoietic stem cell deficiencies in mice lacking c-mpl, the receptor for thrombopoietin. *Proc Natl Acad Sci USA* 1998; 95: 1195–1200.

78 Ihara K, Ishii E, Eguchi M, Takada H, Suminoe A, Good RA, Hara T. Identification of mutations in the c-mpl gene in congenital amegakaryocytic thrombocytopenia. *Proc Natl Acad Sci USA* 1999; 96: 3132–3136.

79 Van den Oudenrijn S, Bruin M, Folman CC et al. Mutations in the thrombopoietin receptor, Mpl, in children with congenital amegakaryocytic thrombocytopenia. *Br J Haematol* 2000; 110: 441–448.

80 Hall JG. Thrombocytopenia and absent radius (TAR) syndrome. *J Med Genet* 1987; 24: 79–83.

81 Ballmaier M, Schulze H, Strauss G et al. Thrombopoietin in patients with congenital thrombocytopenia and absent radii: elevated serum levels, normal receptor expression, but defective reactivity to thrombopoietin. *Blood* 1997; 90: 612–619.

82 Strippoli P, Savoia A, Iolascon A et al. Mutational screening of the thrombopoietin receptor gene (c-mpl) in patients with congenital thrombocytopenia and absent radii (TAR). *Br J Haematol* 1998; 103: 311–314.

Cambridge Books Online © Cambridge University Press, 2009

35

Thrombocytopenias due to bone marrow disorders

Beng H Chong[1] and Colin Chesterman[2]

[1] *University of New South Wales, Sydney, Australia*
[2] *Centre For Thrombosis and Vascular Research, University of New South Wales, Sydney, NSW, Australia*

Thrombocytopenia is a common manifestation in many bone marrow disorders. As bone marrow is the site of platelet production, it is not surprising that bone marrow diseases would cause thrombocytopenia. It is pertinent to review briefly the regulation of platelet production, and how it could be adversely affected by bone marrow diseases.

Regulation of platelet production

Platelets are produced in the bone marrow by cytoplasmic budding of their precursor cell, the megakaryocyte. The megakaryocyte itself is derived from the hematopoietic stem cell by a series of cell proliferation and differentiation processes. These processes are controlled by a network of growth factors, the most important of which is thrombopoietin, TPO[1–4]. These processes also involve a specific program of gene expression mediated by transcription factors such as GATA-1, FOG-1, Fli-1 and NF-F2[5–7]. Mouse knockout studies revealed that disruption of the TPO gene, and genes of these transcription factors, resulted in marked impairment of megakaryocyte development, severe thrombocytopenia and/or serious bleeding[8–12].

TPO exerts its effect by binding to its receptor, c-mpl on megakaryocytes and their precursor cells. TPO-c-mpl interaction induces activation of intracellular signal transduction pathways (Fig. 35.1) including Jak2 / STAT 3/5, protein kinase C (PKC) and MAPK kinase pathways[13]. The signalling pathway activation ultimately leads to activation of specific transcription factors, e.g. GATA-1. The activated factor then translocates to the nucleus and binds to the regulatory region of a target gene involved in megakaryocyte proliferation and differentiation. Many of these target genes are still unknown. However, some are known such as the megakaryocyte-specific genes, GP Ibα, GP IX and GP II b. These are lineage-restricted proteins that are expressed at various stages of megakaryocyte maturation. We, and others, have found that GATA and Ets transcription factors play a critical role in the regulation of these genes[13,14]. However, these transcription factors do not act alone. For example, GATA-1 and FOG-1 interact with one another and with other still unknown factors to form a large protein complex which regulates transcription of megakaryocyte genes. We found that one member of the transcription factor family may replace another member in the regulatory complex. For instance, FOG-2 may replace FOG-1 in its interaction with GATA-1 in the complex that regulates the transcription of the NF-E2 gene[15]. Furthermore, specific factors are only expressed at certain stages of megakaryocyte differentiation (Holmes & BH Chong, unpublished data), suggesting that each factor may be active at a specific phase of megakaryocyte development.

In health, the platelet concentration in the peripheral blood is kept within a narrow range (140×10^9/l – 400×10^9/l). TPO is the key regulator of platelet production. The plasma TPO level is tightly controlled. It varies inversely with the circulating platelet concentration, or more precisely with the platelet/megakaryocyte mass. Hence, the plasma TPO level is increased in patients with thrombocytopenia and vice versa[16,17].

The liver is an important source of TPO production in the body. Other sites of TPO production include the kidneys and the bone marrow stromal cells. The rate of TPO pro-

duction in the liver and kidneys is constant and not influenced by the circulating platelet concentration. Two mechanisms have been postulated for the regulation of blood TPO levels. The first is a passive mechanism in which the circulating TPO level is regulated by its binding to platelets and megakaryocytes and its subsequent degradation inside the cells. When circulating platelets increase, more TPO molecules are taken up by the cells and degraded. The plasma TPO level falls. The reverse occurs when the platelet level decreases. The increase in TPO level is greater in thrombocytopenic states in which the bone marrow megakaryocytes are markedly decreased, as in aplastic anemia[16,17]. In contrast, the circulating TPO is only slightly increased in idiopathic thrombocytopenic purpura (ITP) in which the megakaryocytes are increased or normal[18]. The TPO levels remain elevated in patients with aplastic anemia in remission when the platelet counts and bone marrow megakaryocyte number are normal[17]. The elevated TPO levels in these patients cannot be explained by the passive mechanism. Similarly, the passive mechanism cannot explain the normal plasma TPO levels in patients with reactive thrombocytosis and essential thrombocythemia[19].

It seems most unlikely that the plasma TPO level, which is critically important for maintaining hemostasis and human survival, would be regulated by a passive mechanism alone. Data from Dr Krushansky's and my laboratory (Sungaran et al.) suggest that there is another more precise mechanism which involves TPO gene regulation[20,21]. This mechanism operates locally at the bone marrow, the site of platelet production. The data from both our laboratories show that TPO production by the stromal cells in the bone marrow is under gene regulation[21]. TPO mRNA expression in bone marrow stromal cells varies inversely with the platelet-megakaryocyte mass. It is increased in thrombocytopenic states such as ITP and aplastic anemia[21]. Subsequent studies from my laboratory showed that there is in fact a feedback regulatory loop via the secretion of platelet/megakaryocyte α-granule proteins which modulate the stromal cell TPO gene expression and TPO secretion by these cells[22]. It must be pointed out, however, that the two mechanisms are not mutually exclusive. Both mechanisms may coexist, one as a broadly based regulatory mechanism and the other a more precise local control mechanism at the site of platelet production.

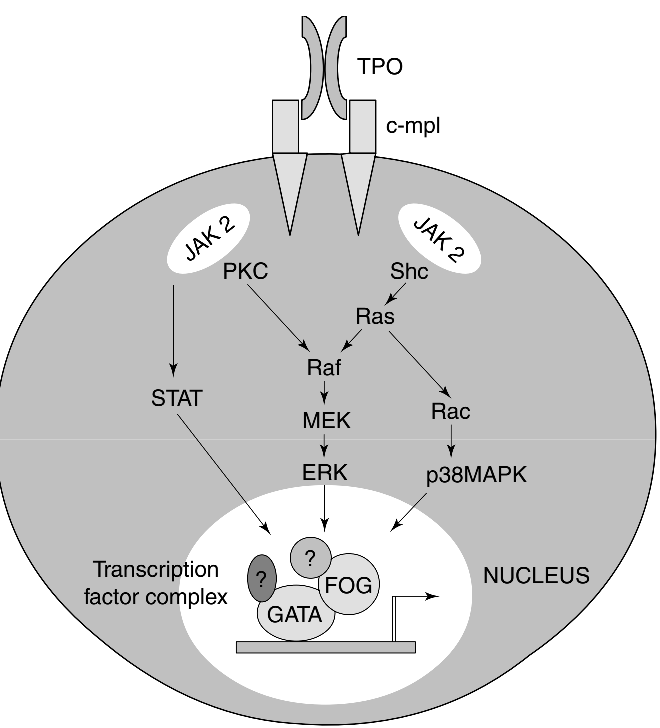


Fig. 35.1. TPO intracellular signalling and gene transcription regulation pathways. This is a diagram showing intracellular cell transduction pathways that become activated when TPO binds to its receptor on a megakaryocytic cell. TPO and c-mpl are in dimeric form. Activation of signal transduction pathway leads to activation and translocation of transcription factors (e.g. GATA-1 and FOG) which bind to the regulatory region of a target gene (e.g. GPIX) and initiate transcription.

Bone marrow disorders that may affect platelet production

Bone marrow disorders could perturb platelet production by several mechanisms. (i) First is depletion of bone marrow hematopoietic stem cells/progenitor cells that give rise to platelets and other mature blood cells by chemotherapeutic drugs, toxic chemicals, ionizing radiation and infective agents. This leads to an aplastic marrow. (ii) Secondly is bone marrow being replaced by cancer or a foreign tissue, resulting not only in thrombocytopenia but also anemia and neutropenia. (iii) Thirdly is replacement of normal hematopoietic stem cells by a malignant/premalignant clone of hematopoietic cells, e.g. in acute leukemias and myelodysplastic syndrome. The abnormal clone has genetic lesions that confer on it a growth or survival advantage, but there is ineffective hematopoiesis resulting in cytopenias (including thrombocytopenia). (iv) Fourthly is a deficiency state, e.g. vitamin B12 or folate deficiency

Cambridge Books Online © Cambridge University Press, 2009

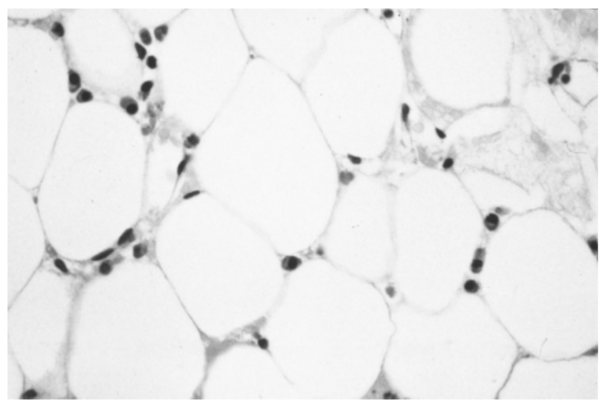


Fig. 35.2 (see also colour plate). A bone marrow trephine biopsy of a patient (male, 25 years of age) with aplastic anemia. It shows predominantly adipose spaces and stromal cells but very few hematopoietic cells.

causing defective cell differentiation and consequently ineffective hematopoiesis/megakaryopoiesis. (v) Fifthly is a defect in regulation of thrombopoiesis.

Thrombocytopenias due to bone marrow disorders will be discussed under the following five headings according to the underlying mechanisms which are responsible for the thrombocytopenia.

Depletion of stem cells: marrow aplasia/ megakaryocytopenia

Aplastic anemia

Aplastic anemia is a heterogenous syndrome characterized by peripheral pancytopenia and bone marrow aplasia. The marrow is devoid of precursors of myeloid, erythroid and megakaryocytic lineages and they are replaced by adipose cells (Fig. 35.2, see colour plate). CD 34+ stem/progenitor cells are markedly reduced in the bone marrow. Bone marrow cells capable of forming granulocyte/monocyte, erythroid and megakaryocyte colonies, CFU-GM, CFU-E and CFU-MK are also decreased[23]. The bone marrow stromal cells are normal. There is no deficiency of hematopoietic growth factors and their levels in plasma, e.g. TPO are compensatorily increased.

Immune mechanism (idiopathic aplastic anemia)

Aplastic anemia can be divided into two types, idiopathic and secondary. Idiopathic aplastic anemia is an uncommon condition which is now widely accepted to be an autoimmune disease[23]. In this condition, there is evidence of suppression of normal hematopoiesis by T lymphocytes mediated by TH-1 type cell activation response, as suggested by increased production of TNF-α, γ- interferon and IL-2. The immune attack causes apoptosis of CD34+ stem/progenitor cells and results in their depletion in the bone marrow[23]. Some secondary aplastic anemias associated with autoimmune disorders such as thymoma and systemic lupus erythematosis (SLE) may share the same T-cell mediated pathophysiology. There may be a genetic predisposition to the development of aplastic anemia. HLA-DR2 occurs twice as frequently in patients with aplastic anemia as in normal individuals[24]. A specific class II haplotype, i.e. DRB 1501 is strongly associated in Japanese patients with cyclosporin-responsive aplastic anemia[25].

Drugs

The commonest cause of bone marrow aplasia is drug. The mechanism may be a direct toxic effect on the stem cells, e.g. alkylating agents, antimetabolites and other cytotoxic drugs or an idiosyncratic (hypersensitivity) reaction. Many drugs have been reported to cause marrow aplasia by an idiosyncratic reaction[26]. The cause-and-effect relationship is only suggestive for some of the reported drugs, but the relationship is more definitive in others such as chloramphenicol, quinacrine, non-steroidal anti-inflammatory drugs (indomethacin, diclofenac, etc.), sulfonamides, hydantoins, pyrazolones, phenothiazines, penicillamine, gold and antithyroid drugs[26]. Incidence of aplastic anemia due to these drugs is largely unknown except for chloramphenicol and quinacrine. The incidence of aplastic anemia caused by these two drugs is estimated to be approximately 1 in 30 000[27,28]. The offending drugs usually cause a generalized marrow aplasia and peripheral pancytopenia. On withdrawal of the offending drug, the marrow usually recovers, but the thrombocytopenia is often the last to do so. Isolated thrombocytopenia due to selective suppression of megakaryocyte may occur with some drugs such as chlorothiazide diuretics[29]. Thrombocytopenia was reported when these drugs were introduced in the 1950s. Thrombocytopenia developed over several weeks, both on initial exposure and on rechallenge. The estimated frequency of thrombocytopenia is one in 15 000 users. There were isolated reports of thrombocytopenia due to diethylstilbestrol caused by a decreased platelet production[30].

Chemicals

Among the chemicals which have been recognized as being toxic to the marrow are benzene, insecticides (pentachlorophenol, lindane and DDT), trinitrotoluene and toluene[31]. These chemicals are used in industry, on the farm and at home. Benzene is used in industry as an organic solvent. It is present in unleaded petrol and tobacco smoke. Benzene and its metabolite *p*-benzoquinone suppress DNA synthesis in hematopoietic pro-

Cambridge Books Online © Cambridge University Press, 2009

genitor cells and inhibit their proliferation[32,33]. These effects may lead to marrow aplasia and subsequent development of myelodysplasia and leukemia. The risk of development of these diseases is higher in individuals who are genetically predisposed. NAD(P)H: quinone oxidoreductase (NQO) plays a critical role in detoxifying benzene metabolites. Chinese workers who were exposed to benzene and had a non-functioning ^{609}C→T polymorphic allele of the NQO1 gene were reported to have an increased incidence of hematologic malignancies[34].

Alcohol

Thrombocytopenia associated with alcohol abuse is usually multifactorial. It may be due to hypersplenism, which is the result of liver cirrhosis, portal hypertension and congestive splenomegaly. Folate deficiency may also contribute to the thrombocytopenia. However, alcohol can cause direct marrow suppression, affecting megakaryocyte maturation resulting in acute thrombocytopenia[35,36]. Ingestion of alcohol for 5–10 days is required for sustained thrombocytopenia. After cessation of alcohol consumption, the thrombocytopenia resolves within 5–21 days. However, since not every alcoholic develops thrombocytopenia, additional factors may need to be present for thrombocytopenia to occur.

Radiation

Acute exposure to radiation may lead to bone marrow suppression, the severity of which is dependent on the radiation dose. A dose of 1–2.5 Gy will result in leukopenia, 4.5 Gy death in 50% of the exposed individuals and >10 Gy universal death[37] unless the patients receive bone marrow transplantation and supportive care. Low doses such as those given during cancer therapy can also cause marrow aplasia. The degree of marrow hypoplasia depends, of course, on the dose, duration and site of radiation exposure and also on whether the patient has had prior chemotherapy. Marrow aplasia may recover but myelodysplasia and leukemia may subsequently occur[38].

Viral infection

There is a close association between viral infection, specifically hepatitis and Epstein–Barr virus (EBV) infection, and the subsequent development of aplastic anemia. Most cases of hepatitis-related aplastic anemia are caused by non-A, non-B and non-C hepatitis, although a few patients have been reported to be due to hepatitis A and B[39]. In many patients, aplastic anemia occurs 1–3 months or occasionally even a year after the onset of infection when the liver disease is resolving or has resolved. At present, it is unclear whether the marrow aplasia is the result of viral

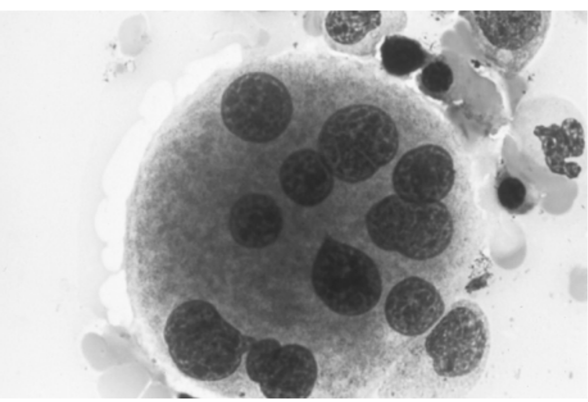


Fig. 35.3 (see also colour plate). This shows an abnormal megakaryocyte with dysplastic changes in a patient with HIV-1 infection and thrombocytopenia. It is a huge cell with nuclear fragmentation.

infection of the hematopoietic stem/progenitor cells or marrow injury by the host immune system. However, there is no association between aplastic anemia and hepatitis C, which can cause thrombocytopenia by hypersplenism and other mechanisms. Pancytopenia is frequently encountered in HIV(human immunodeficiency virus) infection, particularly in the late stages of the disease but the marrow is often hypercellular and the pancytopenia is due to ineffective hematopoiesis as a result of HIV infection of bone marrow stromal cells[40,41]. Occasionally, marrow hypoplasia is seen. This is attributed to marrow suppression by infection and drugs used to control viral replication.

Isolated thrombocytopenia is often noted in viral infection and this is believed to be due to at least two different mechanisms, namely infection of megakaryocytes (Fig. 35.3, see colour plate) and immune peripheral platelet destruction. Thrombocytopenia due to the former mechanism has been reported in measles, HIV (human immunodeficiency virus), varicella, mumps, Epstein-Barr virus, rubella, cytomegalovirus, parvovirus and dengue infection[42]. In these infections, bone marrow biopsy may show nuclear and cytoplasmic vacuolation in megakaryocytes under light microscopy and inclusion bodies on electron microscopy. Interestingly, in HIV infection, the virus enters the megakaryocyte progenitors at a stage when they express CD 4 and other chemokine coreceptors like CXCR4[43]. Infection of megakaryocytes leads to ineffective thrombopoiesis. The thrombocytopenia is, in part, also due to immune platelet destruction, particularly in HIV infection as the thrombocytopenia frequently responds to corticosteroid, high dose IgG infusion and splenectomy[44,45].

Cambridge Books Online © Cambridge University Press, 2009

Miscellaneous causes

This group of secondary aplastic anemia is likely to have an immune etiology such as aplastic anemia seen in the occasional patients with systemic lupus erythematosus[46]. These patients usually have antibody or suppressive T lymphocytes that inhibit hematopoiesis. Although immune etiology is also likely to be present in rheumatoid arthritis patients with coexisting aplastic anemia, it is difficult to exclude the possibility that drugs used to treat the condition such as gold, methotrexate and D-penicillamine, may also contribute to the marrow aplasia. In eosinophilic fasciitis, the immune etiology of the associated aplastic anemia is even less certain as the pancytopenia is usually unresponsive to immunosuppressive therapy. However, occasional patients have partially or completely recovered with antithymocyte globulin (ATG) and/or cyclosporin[47]. Aplastic anemia is also known to be associated with pregnancy, in which case the disease usually improves with termination of pregnancy or after delivery[48]. It may recur with subsequent pregnancies. However, the condition may also progress even after delivery of the baby.

Treatment of aplastic anemia

If there is an identifiable cause, it should be removed or treated, e.g. cessation of the offending drug, termination of pregnancy or treatment of the underlying infection (e.g. treatment of HIV-1 infection with zidovudine). Supportive treatment is also important and this includes platelet transfusion for bleeding due to thrombocytopenia, packed cell transfusion for anemia and antibiotic treatment for bacterial infection.

Specific treatment is either allogeneic bone marrow transplant (replenishment of hematopoietic stem cells), usually from a histocompatible sibling, or abrogating the immune attack by immunosuppressive therapy[49]. The response to allogeneic transplantation, particularly in children, is very favourable. Long-term survival rates (after 5 years) vary from 75–90%. Acute transplant mortality has decreased substantially and graft-vs.-host disease(GVHD) is now better controlled[50]. GVHD is still a limiting factor in the success of bone transplantation, especially in adult patients as the incidence and severity of GVHD increase with age. Patients without a compatible marrow donor and those who are unsuitable for transplantation, are treated with immunosuppressive therapy using cyclosporin or antithymocyte globulin (ATG)/antilymphocyte globulin (ALG). Patients respond better to cyclosporin plus ATG/ALG than either drug alone. A response rate of 70–80% can be expected in patients receiving the combined therapy with a survival rate at 5 years of 90% among the responders[51].

Acquired amegakaryocytic thrombocytopenic purpura (AATP)

This is a rare disorder characterized by severe thrombocytopenia and absence of megakaryocytes in the bone marrow. Bone marrow aspiration or trephine biopsy shows a marked decrease or absence of megakaryocytes[52] with normal erythropoiesis and granulopoiesis. An antiplatelet antibody is usually not detected. AATP is analogous to pure red cell aplasia in which there is a severe anemia and absence of erythroid precursors in the bone marrow. The pathophysiology of AATP is at present unclear. It may have different underlying mechanisms. Ex vivo culture of the patient's bone marrow cells shows no or very few CFU-MK (colony forming unit-megakaryocyte). Sera or T lymphocytes from patients with AATP have been shown to suppress megakaryocyte colony formation when added to in vitro culture of normal bone marrow[52–55]. These studies suggest a humoral or T suppressor lymphocyte-mediated inhibition of megakaryopoiesis, although an intrinsic CFU-MK defect may also be present.

The pathophysiology of AATP is similar to that observed in aplastic anemia. There have been cases of AATP who progressed rapidly to aplastic anemia[56] and others in whom the disease transformed to acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS)[57]. Like aplastic anemia and pure red cell aplasia, AATP responds well to immunosuppressive therapy including ATG/ALG, cyclosporin and cyclophosphamide[58,59]. There is, as yet, no report of treatment of AATP with bone marrow transplantation.

Paroxysmal nocturnal hemoglobinuria (PNH)

PNH is a disease related to aplastic anemia. The main clinical manifestations of this disease are intravascular hemolysis and increased tendency to thrombosis[60]. Thrombocytopenia may also occur in some patients with PNH. PNH and aplastic anemia are in the same spectrum of diseases as some patients with PNH may transform to aplastic anemia[61] and vice versa. PNH is caused by somatic mutations in the PIG-A (phosphatidyl inositol glycan complementation Group A) gene leading to deficiency of glycosyl phosphatidyl inositol (GPI)-anchored proteins on red cells and other blood cell surfaces[62]. As a result, red cells are very susceptible to lysis by terminal complement components, C5–C9 and this explains the intravascular hemolysis in PNH[62]. The mechanism causes platelet activation and consequently thrombosis in this condition.

The anemia and thrombosis in PNH are usually treated with blood transfusion and anticoagulant therapy. PNH patients with aplastic anemia or severe cytopenia may be treated like non-PNH patients who have aplastic anemia

Cambridge Books Online © Cambridge University Press, 2009

with immunosuppressive therapy or bone marrow transplantation as discussed above.

Fanconi anemia (FA)

Fanconi Anemia (FA) is an autosomal recessive congenital blood disorder, which frequently progresses to aplastic anemia in childhood but occasionally in adulthood[63]. The patients with FA also have a predisposition to development of AML, MDS and other cancers[64]. They frequently have multiple physical abnormalities such as hyperpigmentation, short stature, microophthalmia and microcephaly. Recently, two FA genes have been isolated and specific mutations in these genes have been identified[65,66]. The genetic defects are responsible for the spontaneous chromosomal breakage, which predispose them to the development of aplastic anemia, AML, MDS and other cancers. For laboratory diagnosis of FA, patient lymphocytes are cultured in the presence of DNA cross-linking agents such as mitomycin C and diepoxybutane (DEB)[67,68]. Cells from FA patients often have spontaneous chromosomal breakage which is increased substantially on exposure to these bifunctional alkylating agents.

The anemia and other cytopenias in FA patients are managed with supportive care including transfusion with packed cells and platelets. Androgen and growth factors such as erythropoietin, G–CSF and GM–CSF may also be helpful. For those who develop aplastic anemia, allogeneic bone marrow transplantation should be considered if a compatible donor is available[69]. Patients with FA often have endocrine dysfunction such as growth hormone deficiency and hypothyroidism which may need treatment with the appropriate hormone replacement.

Depletion of stem cells – bone marrow replacement by neoplasm or foreign tissues

Bone marrow infiltration by malignancy

The thrombocytopenia in hematologic malignancy (e.g. multiple myeloma and lymphoma) and metastatic non-hematologic cancer (e.g. prostate, breast, lung and renal cancer) (Fig. 35.4, see colour plate), often occurs together with anaemia and neutropenia (pancytopenia). The pancytopenia is due to suppression of normal hematopoiesis by marrow infiltration by cancer or foreign tissues, e.g. myelofibrosis. Inhibition of the normal hematopoietic clone of cells may be simply due to the overcrowding effect but secretion of an inhibitory factor by the malignant cells has been suggested. Chemotherapy and DIC may also contribute to the thrombocytopenia in this group of patients.

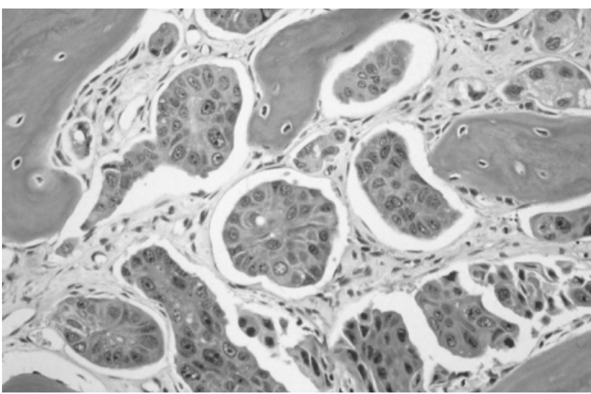


Fig. 35.4 (see also colour plate). A bone marrow trephine biopsy of a patient with metastatic breast cancer showing bone marrow infiltration by malignant cells. The cancer cells are arranged in a gland-like formation (acini).

Ineffective thrombopoiesis: megaloblastic anemia

Folate/vitamin B_{12} deficiency

Megaloblastic anemias are a group of bone marrow disorders showing typical blood and bone marrow abnormalities due to impaired DNA synthesis[70]. The main causes of megaloblastic anemia are vitamin B_{12} and folate deficiency. In these deficiency states, there is a decrease in deoxythymidine triphosphate (dTTP) due to the so-called 'methylfolate trap'[71]. This leads to a slowing of DNA replication and consequently impairment of cell development affecting all hemapoietic lineages. The hypercellular bone marrow shows cells with nuclear maturation lagging behind cytoplasmic. As a result the cells acquire more cytoplasm, and become larger than normal. These changes are most prominent in the erythroid precursors. The abnormally large erythroblasts are termed megaloblasts (Fig. 35.5, see colour plate)[70]. The megaloblastic changes also affect granulocyte precursors and megakaryocytes, which can be abnormally large with a bizarre nucleus containing numerous unconnected lobes[72]. In the peripheral blood, the red cells display marked variation in size but are often large and oval. The neutrophils are hypersegmented and the platelets are small[73]. The ineffective hematopoieis leads to pancytopenia. In severe vitamin B12 deficiency, the platelets are not only reduced in number but can also be functionally abnormal[74].

The commonest cause of folate deficiency is inadequate dietary intake of folate as the hepatic store of folate is small[75]. This occurs frequently in the elderly, the poor, the alcoholic and those with increased folate requirement

Cambridge Books Online © Cambridge University Press, 2009

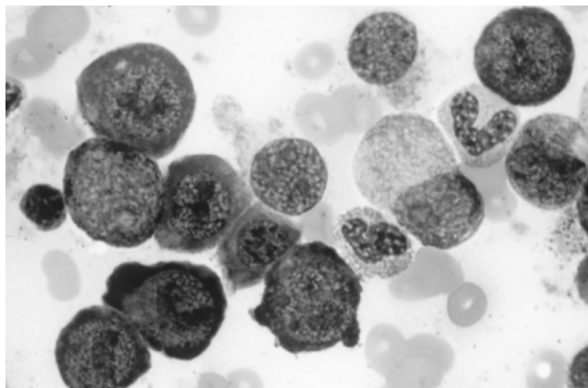


Fig. 35.5 (see also colour plate). A bone marrow smear of a patient (female, aged 76) with pernicious anemia. It shows florid megaloblastic changes with many megaloblasts and a giant band form.

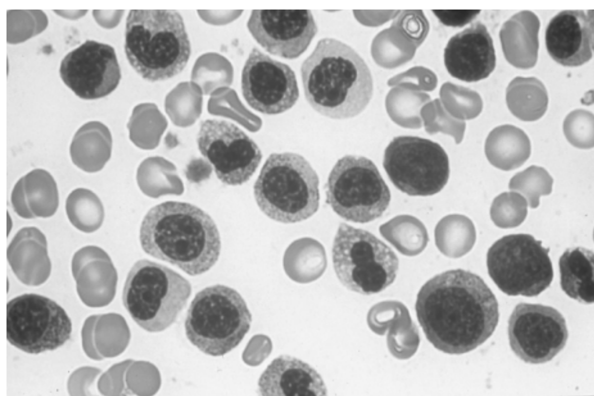


Fig. 35.6 (see also colour plate). A bone marrow smear of a patient (male, aged 64) who had myelodysplastic syndrome. It shows dysplastic changes in granulocyte precursors, namely hypogranular forms and Pelger–Huet anomaly.

such as pregnant women, patients on hemodialysis and those with chronic hemolytic anemia. Besides poor intake, folate malabsorption, e.g. in patients with tropical and non-tropical sprue, celiac disease, regional enteritis and other intestinal disorders, can also cause folate deficiency. Red cell folate concentration is more reliable than serum folate level for the laboratory diagnosis of folate deficiency as it is less variable and correlates better with the tissue folate status[76]. Folate deficiency is treated with 1–5 mg of folate orally per day. Therapeutic effect is usually achieved even in those with folate malabsorption.

The principal cause of vitamin B_{12} is pernicious anemia (PA) which is an autoimmune disease[77]. In this disease, there is a gastric mucosa defect causing achlorhydria and decreased production of intrinsic factor (IF), which is essential for vitamin B_{12} absorption in the ileum. Patients with PA also produce antibodies to IF and IF-B_{12} complex which inhibit the absorption of vitamin B_{12}. There is an association of PA with a number of autoimmune diseases such as thyroid diseases, type I diabetes mellitus, Addison disease, ulcerative colitis and vitiligo[78]. It occurs more commonly in Northern Europeans and in individuals with HLA types A2, A3, B7 and B12 and blood group A[79,80]. Vitamin B_{12} malabsorption is also seen after total or partial gastrectomy, patients with intestinal diseases (see above), blind loop syndrome and fish tape worm infestation (*Diphyllobothrium latum*). A low serum cobalamin level in the appropriate clinical setting indicates the presence of vitamin B_{12} deficiency. The diagnosis of PA requires in addition the detection of IF antibodies and /or a positive Schilling test. Treatment consists of parenteral administration of cyano- or hydroxocobalamin for life, e.g. in patients with PA or until the cobalamin malabsorption is corrected.

Ineffective thrombopoiesis due to an abnormal stem cell clone

Myelodysplastic syndrome (MDS)

MDS is a common bone marrow disorder among the elderly. It is characterized by expansion of a premalignant stem cell clone, ineffective haematopoiesis and peripheral cytopenia. It consists of heterogeneous conditions with different genetic, biological, morphologic (Fig. 35.6, see colour plate) and clinical features and with varying propensity of progression to acute myeloid leukemia (AML)[81]. In USA, the incidence is 3.5 per 100 000 at age of 50 years, and it increases with age to 15 per 100 000 at age of 70 and 35 per 100 000 at age of 90[82]. The French–American–British (FAB) classification divides MDS into five different conditions as shown in Table 35.1[83]. Dissatisfaction with this purely morphologic classification has recently led to a new classification by the WHO[84], attempting to take into consideration the genetic and biological differences among the subtypes.

The pathogenesis of MDS is still unclear. There is now strong evidence that AML and MDS are pathophysiologically linked. Several etiological factors contribute to the development of MDS/AML.

Genetic predisposition

There is an increased incidence of MDS and AML in families with inherited constitutional genetic defects such as Fanconi anemia, neurofibromatosis type1, Schwachman–Diamond syndrome and familial platelet disorder with leukemia (FPD/AML)[85]. The basis for the increased propensity to MDS/AML is defective DNA repair in Fanconi anemia

Cambridge Books Online © Cambridge University Press, 2009

Table 35.1. FAB classification of myelodysplastic syndrome[83]

Refractory anemia (RA)	Cytopenia of one PB lineage; normo- or hypercellular marrow with dysplasia: <1% PB blasts and <5% BM blasts
Refractory anemia with ringed sideroblasts (RARS)	Cytopenia, dysplasia and the same % blast involvement in BM and PB as RA. Ringed sideroblasts account for >15% of marrow nucleated cells
Refractory anemia with excess blasts (RAEB)	Cytopenia of two or more PB lineages; dysplasia involving all three lineages; <5% PB blasts and 5–20% BM blasts
Refractory anemia with excess blasts in transformation (RAEB-T)	Hematologic features identical to RAEB >5% blasts in PB or 21–30% blasts in BM or the presence of Auer rods in the blasts
Chronic myelomonocytic leukemia (CMML)	Monocytosis in PB ($>1 \times 10^9$/l); <5% blasts in PB and up to 20% BM blasts

Notes:
PB = peripheral blood; BM = bone marrow.

and deregulation of the RAS signal transduction pathway in neurofibromatosis[63,86]. An association of NQ01 gene polymorphism with MDS/AML has also been reported recently[34] (see above).

Environmental carcinogens

Recent epidemiologic studies have revealed an association between MDS and smoking/exposure to toxic agents, e.g. petroleum products, organic solvents, fertilizers, arsenic, thallium, etc.[87].

Cytogenetic abnormalities

Gain or losses of specific chromosomal regions, e.g. 7q-, 5q-, 20q11–12, trisomy 8, 12p- and 3q-, are frequently detected in patients with MDS[88].

Genetic defects

Besides the inherited genetic changes described above, acquired mutations in the RAS, p53 and FLT3 genes have been reported in MDS patients[86,89,90]. Progressive methylation and inactivation of cell cycle regulatory genes e.g. $p15^{INK4b}$ have been found in some patients with MDS[91]. Erythropoietin-induced activation of the STAT 5 signal transduction pathway has been found to be impaired in patients with MDS[92] and this is believed to be responsible for the refractory anemia seen in these patients.

These findings suggest that the disease develops over a period of time by a multistep genetic progression to MDS and then AML. At least in some patients, there is a strong inherited predisposition, e.g. defects in DNA repair genes, mutation in AML1 gene or NQO1 polymorphism with reduced ability to detoxify benzene metabolites[34,63,85]. Subsequently insult by environmental carcinogens[87] and genomic instability lead to stepwise gains/losses of specific chromosomal regions (described above) or chromosome translocations /inversion e.g. t(3; 21), t(8; 21), t(12; 21) and inv[16], that ultimately result in further genetic changes[88] that may involve a tumour suppressor gene (e.g. p53), a gene in a signalling pathway (e.g. RAS) or a cell cycle regulatory gene, e.g. $p15^{INK4b}$ [89–92]. From these genetic changes, a premalignant hematopoietic stem cell clone with growth or survival advantage, emerges and expands. MDS is established. With further genetic changes, it progresses to AML.

Role of aberrant cytokine production and apoptosis in development of MDS

Several studies have reported elevated levels of tumour necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β) and interleukin-1 β (IL-1β). Increased IL-1β is believed to play a role in supporting the expansion of the premalignant clone. Excessive TNF-α may contribute to the accelerated apoptosis and ineffective hematopoiesis in MDS. There could be additional reasons for the excessive apoptosis such as perturbed adhesive interactions between hematopoietic and stromal cells[88].

Immune mechanisms in MDS

It has been postulated that transformation of normal to aberrant stem cells induces an autoimmune T cell response. The MDS stem cell clone is more resistant than the normal clone to the immune attack and expands at the expense of the normal clone[88]. The data in support of an autoimmune process in MDS are:

(i) the presence of skewed T cell receptor V-β repertoire suggesting T cell clonal expansion in MDS[88];
(ii) MDS T cells inhibit CFU-E and CFU-GM in vitro[93,94]; and
(iii) immunosuppressive therapy with ATG and cyclosporin improve cytopenia in MDS[94,95].

Cambridge Books Online © Cambridge University Press, 2009

In summary, MDS arises after a series of genetic changes that confer growth/survival advantage on an aberrant hematopoietic stem cell clone. An autoimmune T cell response which attacks the normal stem cell clone more severely, further allows expansion of the aberrant clone at the expense of the normal stem cell clone. Excessive apoptosis of the progeny of the aberrant stem cells, mediated by increased TNF-α and other mechanisms, leads to intramedullary death of the mature blood cells before their release into the blood stream and pancytopenia.

Treatment

Younger patients with MDS have been treated with curative intent using allogeneic bone marrow transplantation[96]. The results have been variable and are poor for those with unfavourable cytogenetics and other features. Consolidation treatment with autologous bone marrow transplant after intensive chemotherapy may lead to long-term survival in a small proportion of high risk MDS patients[88]. Treatment with low dose 5-azacytidine has delayed progression to AML and death in also a small proportion of high risk patients[97]. The treatment in the majority of patients who are elderly is largely supportive care with blood transfusion for anemia, platelet transfusion for thrombocytopenia and bleeding and antibiotic therapy for infection. Recently, erythropoietin and granulocyte colony-stimulating factor have been shown to act synergistically to increase Hb levels and to lengthen the time interval between transfusions[98]. Immunosuppressive therapy with ATG or cyclosporin may improve the cytopenia in MDS, especially in patients with the subtype refractory anemia[88,94,95].

Acute leukemia (AL)

In AL, normal hematopoietic precursors in the bone marrow are replaced by a malignant clone of cells which fail to differentiate into mature blood cells resulting in pancytopenia (thrombocytopenia included). The block in differentiation occurs at a very early stage of myeloid maturation in AML or lymphocytic differentiation in acute lymphoblastic leukemia. A subtype of AML, acute promyelocytic leukemia (APML) deserves a special mention. In APML, the maturation arrest is at a later stage (the promyelocyte stage). The promyelocytes possess abundant cytoplasmic primary granules, which contain myeloperoxidase, proteases (e.g. cathepsin G) and other procoagulant materials. Death of the leukemic cells and release of the granules lead to disseminated intravascular coagulation (DIC)[99]. Before the advent of all-*trans* retinoic acid (ATRA) for its treatment, many patients with APML died of bleeding because of DIC[99]. Unlike chemotherapeutic agents, which cause massive leukemic cell kill and sudden outpour of granular constituents into the circulation causing DIC, ATRA eradicates the malignant clone by inducing cell differentiation, avoiding the abrupt leukemic cell death and DIC[100]. With ATRA treatment, serious bleeding and other toxicity could be avoided or minimized. However, the remission in patients treated with ATRA alone is usually short lived. The optimal treatment of APML is a combination of ATRA and chemotherapy. Detailed discussion on leukemia and its treatment is beyond the scope of this chapter. Readers are referred to specific reviews on this topic[99–102].

Disorders of platelet regulation

Thrombopoietin levels in thrombocytopenic states

Knockout studies showed that, in mice in which the TPO gene or its receptor, m-pl gene was deleted, the circulating platelet levels were extremely low, only10–20% of the wild-type or control animal[8,103]. This suggests that TPO is responsible for the regulation of 80–90% of platelet production. A decrease in TPO or m-pl caused by a genetic defect, would result in thrombocytopenia. No congenital thrombocytopenic disorders with decreased blood TPO level have yet been identified. On the contrary, elevated TPO levels were found in congenital thrombocytopenic disorders including congenital amegakaryocytic thrombocytopenia, thrombocytopenia with absent radii, Fanconi's anemia and Schwachman–Diamond syndrome[104–106]. Raised plasma TPO levels are also observed in the acquired thrombocytopenic disorders in adults such as aplastic anemia, thrombocytopenia associated with chemotherapy, leukemia and other primary bone marrow disorders, and idiopathic thrombocytopenic purpura (ITP)[16–18]. These findings indicate that the altered TPO levels are the consequence, and not the cause of the thrombocytopenia.

TPO deficiency: liver disease

The liver is the major source of TPO production in the body. Thrombocytopenia is a common manifestation of liver disease. A known cause of thrombocytopenia in liver disease is platelet sequestration in the enlarged spleen (hypersplenism). Is decreased hepatic TPO production another cause? The plasma TPO levels in patients with chronic hepatitis and many patients with cirrhosis are elevated, particularly if they are not thrombocytopenic[107,108]. The reason for the raised TPO levels is unclear. It may pos-

Cambridge Books Online © Cambridge University Press, 2009

sibly be related to the chronic inflammatory process in the liver. However, the TPO level decreases with the severity of the disease and patients with end-stage liver cirrhosis have slightly decreased TPO levels[109,110]. Liver TPO mRNA levels are also decreased in these patients[110]. The blood TPO levels and platelet counts return to normal after liver transplant[111], suggesting that decreased TPO production by the liver does play a role in the thrombocytopenia of liver disease.

Renal disease

Although the kidney is also a prominent source of TPO, the circulating TPO levels remain normal in patients with end-stage renal failure and thrombocytopenia is not a common clinical finding in these patients.

Autoimmune thrombocytopenia

A decreased TPO blood level as a result of anti-TPO antibody, may also be a cause of thrombocytopenia. Human subjects and animals receiving recombinant TPO have been reported to develop anti-TPO antibodies[112–114], decrease in serum TPO levels and severe thrombocytopenia. These data suggest that, in some patients, chronic thrombocytopenia associated with low plasma TPO levels may be due to anti-TPO antibodies.

Decreased sensitivity to TPO: defects in c-mpl or signalling pathways

Congenital amegakaryocytic thrombocytopenia (CAMT)

CAMT is a rare cause of congenital thrombocytopenia in childhood. It is characterized by isolated thrombocytopenia and absence of megakaryocytes in the bone marrow. The serum TPO levels in these patients are increased[106]. Bone marrow CD 34+ progenitor cells from patients with CAMT failed to differentiate into megakaryocytes. Ihara et al.[115] and van den Oudenrijn et al.[106] have found point mutations and/or a deletion in the coding regions of c-mpl in patients with CAMT[115,106]. These mutations lead to premature stop codon or loss of a splice site, which, if translated, will result in a non-functional receptor. The c-mpl mRNA levels in marrow mononuclear cells are usually very low or undetectable, suggesting defective translation or unstable transcripts in this disorder.

Patients with CAMT are compound heterozygote for two mutations or homozygote for one mutation in the c-mpl gene[106,115]. Their parents or siblings who carry one affected allele had normal megakaryocytes, platelets and TPO levels, suggesting that only one normal c-mpl allele is required for normal megakaryocytopoiesis. As TPO also has an effect on the survival of hematopoietic stem cells and early progenitor cells of other lineages, patients with CAMT often progress to develop, in addition, neutropenia, anemia and ultimately bone marrow failure.

Thrombocytopenia with absent radii (TAR)

TAR is a rare congenital thrombocytopenic disorder characterized by absent megakaryocytes in the bone marrow and absence of radii in both arms. The blood TPO level is increased and c-mpl is normal[104,105]. It appears that there is a defect in the signal transduction pathway of c-mpl that is responsible for the abnormal megakaryocyte proliferation and differentiation in TAR syndrome[104,105].

Cyclic thrombocytopenia

Cyclic thrombocytopenia is a rare disorder in which the platelet counts fluctuate from $<20\times10^9$/l to as high as 1000×10^9/l[116]. It is commoner in women than in men. In women, the platelet cycles usually occur in synchrony with menstruation[117]. Different mechanisms have been postulated for the platelet fluctuation. In some patients, there is a cyclical failure of megakaryocytopoiesis with a decrease in CFU-meg, bone marrow megakaryocytes and platelets, in that sequential order[118]. However, the reason for the cyclical failure of thrombopoiesis is still unknown. In some cases, an immune process may cause cyclical destruction of platelets[119]. It has been suggested that, in women in whom the cyclic thrombocytopenia occurs in phase with the menstrual cycle, the high estrogen level in the late luteal phase enhances macrophage Fcγ receptor in the spleen and causes increased platelet clearance[117].

Acknowledgements

The authors wish to thank Ms Gillian Rozenberg for her contribution in producing bone marrow photomicrographs (Fig. 35.2–35.6) and Dr T.B. Keng for his help in manuscript preparation.

References

1 DeSauvage FJ, Hass PE, Spencer SD et al. Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-MPL ligand. *Nature* 1994; 369: 533–538.

Cambridge Books Online © Cambridge University Press, 2009

2 Lok S, Kaushansky K, Holly RD et al. Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo. *Nature* 1994; 369: 565–567.
3 Bartley TD, Bogenberger J, Hunt P. Identification and cloning of a megakaryocyte growth and development factor that is a ligand for the cytokine receptor Mpl. *Cell* 1994; 77: 117–124.
4 Tao H, Gaudry L, Rice A, Chong B. Megakaryocytopoiesis: cord blood is better than bone marrow for generating megakaryocytic progenitor cells. *Exp Haematol* 1999; 27: 293–301.
5 Shivdasani R, Orkin SH. The transcriptional control of hematopoiesis. *Blood* 1996; 87: 4025–4039.
6 Tsang AP, Visvader JE, Turner CA et al. FOG, a multitype zinc finger protein, acts as a cofactor for transcription factor GATA-1 in erythroid and megakaryocytic differentiation. *Cell* 1997; 90: 109–119.
7 Athanasiou M, Clausen P, Mavrothalassitis G, Zhang X, Watson D, Blair D. Increased expression of the Ets-related transcription factor Fli-1/ERGB correlates with and can induce the megakaryocytic phenotype. *Cell Growth Diff* 1996; 7: 1525–1534.
8 Gurney AL, Carver-Moore K, de Sauvage FJ, Moore MW. Thrombocytopenia in c-mpl deficient mice. *Science* 1994; 265: 1445–1447.
9 Pevny L, Simon MC, Robertson E et al. Erythroid differentiation in chimeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* 1991; 349: 257–260.
10 Tsang AP, Fujiwara Y, Hom DB, Orkin SH. Failure of megakaryopoiesis and arrested erythropoiesis in mice lacking the GATA-1 transcriptional cofactor FOG. *Genes Dev* 1998; 12: 1176–1188.
11 Spyropoulos D, Pharr P, Lavenburg K et al. Hemorrhage, impaired haematopoiesis, and lethality in mouse embryos carrying a targeted disruption of the Fli1 transcription factor. *Mol Cell Biol* 2000; 20: 5643–5652.
12 Shivdasani RA, Rosenblatt MF, Zucker-Franklin D et al. Transcription factor NF-E2 is required for platelet formation independent of the actions of thrombopoietin/MGDF in megakaryocyte development. *Cell* 1995; 81: 695–704.
13 Liu R, Fan C, Gracia R, Jove R, Zuckerman K. Constitutive activation of the JAK2/STAT 5 signal transduction pathway correlates with growth factor independence of megakaryocytic leukaemic cell lines. *Blood* 1999; 93: 2369–2379.
14 Doubeikovski A, Uzan G, Doubeikovski Z et al. Thrombopoietin-induced expression of the glycoprotein IIb gene involves the transcription factor PU.1/Spi-1 in UT7–Mpl cells. *J Biol Chem* 1997; 272: 24300–24307.
15 Holmes M, Turner J, Fox A, Chisholm O, Crossley M, Chong B. hFOG-2, a novel zinc finger protein, binds the co-repressor mCtBP2 and modulates GATA-mediated activation. *J Biol Chem* 1999; 274: 23491–23498.
16 Hou M, Andersson PO, Stockelberg D, Mellqvist UH, Ridell B, Wadenvik H. Plasma thrombopoietin levels in thrombocytopenic states: implication for a regulatory role of bone marrow megakaryocytes. *Br J Haematol* 1998; 101: 420–424.
17 Schrezenmeier H, Griesshammer M, Hornkohl A et al. Thrombopoietin serum levels in patients with aplastic anaemia: correlation with platelet count and persistent elevation in remission. *Br J Haematol* 1998; 100: 571–576.
18 Porcelijn L, Folman CC, Bosssers B et al. The diagnostic value of thrombopoietin level measurements in thrombocytopenia. *Thromb Haemost* 1998; 79: 1101–1105.
19 EL-Kassar N, Hetet G, Briere J, Grandchamp B. Clonality analysis of hematopoiesis and thrombopoietin levels in patients with essential thrombocythaemia. *Leukemia Lymphoma* 1998; 30: 181–188.
20 McCarty JM, Sprugel KH, Fox NE, Sabath DE, Kaushansky K. Murine thrombopoietin mRNA levels are modulated by platelet count. *Blood* 1995; 86: 3668–3675.
21 Sungaran R, Markovic B, Chong BH. Localisation and regulation of thrombopoietin mRNA expression in human kidney, liver, bone marrow and spleen using in situ hybridization. *Blood*; 1997; 89: 101–107.
22 Sungaran R, Chisholm OT, Markovic B, Khachigian LM, Tanaka Y, Chong BH. The role of platelet a-granular proteins in the regulation of thrombopoietin messenger RNA expression in human bone marrow stromal cells. *Blood* 2000; 95: 3094–3101.
23 Young NS, Maciejewski JP. The pathophysiology of acquired aplastic anaemia. *N Engl J Med* 1997; 336: 1365–1372.
24 Nimer SD, Ireland P, Meshkinpour A, Frane M. An increased HLA DR2 frequency is seen in aplastic anaemia patients. *Blood* 1994; 84: 4257–4261.
25 Nakao S, Takamatsu H, Chuhjo T et al. Identification of a specific HLA class II haplotype strongly associated with susceptibility to cyclosporine-dependent aplastic anaemia. *Blood* 1994; 84: 4257–4261.
26 Adamson J, Erslev AJ. Aplastic anaemia, In Williams WJ, Beutler E, Erslev AJ, Litchman MA, eds. *Hematology*. New York: McGraw-Hill; 1990: 158–174.
27 Custer RP. Aplastic anaemia in soldiers treated with Atabrine (quinacrine). *Am J Med Sci* 1969; 212: 211.
28 Smick K, Condit PK, Proctor RL, Sutcher V. Fatal aplastic anemia: an epidemiological study of its relationship to the drug chloramphenicol. *J Chronic Dis* 1964; 17: 899.
29 Okafor KC, Griffin C, Ngole PM. Hydrochlorothiazide-induced thrombocytopenic purpura. *Drug Intell Clin Pharm* 1986; 20: 60–61.
30 Nachtsheim DA, McPherson RA, Pollen JJ, Schmidt JD. Thrombocytopenia during diethylstilbestrol diphosphate infusion for carcinoma of the prostate. *Prostate* 1980; 1: 105–109.
31 Rugman FP, Cosstick R. Aplastic anaemia associated with organochloride pesticide (Lindane). Case reports and review of evidence. *J Clin Pathol* 1990; 43: 98–101.
32 Kalf GF, Rushmore T, Snyder R. Benzene inhibits RNA synthesis in mitochrondria from liver to bone marrow. *Chem Biol Interact* 1982; 42: 353.
33 Thnek A, Hogstedt B, Orofsson T. Mechanism of benzene toxicity: effects of benzene and benzene metabolites on bone

Cambridge Books Online © Cambridge University Press, 2009

marrow cellularity, number of granulocytic stem cells and frequency of micronuclei in mice. *Chem Biol Interact* 1982; 39: 129.
34 Rothman N, Smith MT, Hayes RB et al. Benzene poisoning, a risk factor for haematologic malignancy, is associated with the NQ01 609 C→T mutation and rapid fractional excretion of chlorzoxazone. *Cancer Res* 1997; 57: 2839–2842.
35 Cooper GW, Dinowitz, Cooper B. The effects of administration of ethyl alcohol to mice on megakaryocyte and platelet development. *Thromb Haemost* 1984; 52: 11–14.
36 Levine RF, Spivak JL, Meagher RC, Sieber F. Effect of ethanol on thrombopoiesis. *Br J Haematol* 1986; 62: 345–354.
37 Mettler FA, Moseley RDJ. *Medical effects of ionizing radiation.* New York: Grune & Stratton; 1985: 1–283.
38 Court-Brown WM, Doll R. Leukaemia and aplastic anaemia in patients irrradiated for ankylosing spondylitis. *Med Res Council Special Report* 295, London: HM Stationery office; 1957.
39 Brown KE, Tisdale J, Dunbar CE, Young NS. Hepatitis-associated aplastic anaemia. *N Engl J Med* 1997; 336: 1059–1064.
40 Najean Y, Rain JD. The mechanism of thrombocytopenia in patients with HIV infection. *J Lab Clin Med* 1994; 123: 415–420.
41 Kulkosky J, Bouhamdan M, Geist A, Nunnari G, Phinney DG. Pathogenesis of HIV-1 infection within bone marrow cells. *Leuk Lymphoma* 2000; 37: 497–515.
42 Kunzi MS, Groopman JE. Identification of a novel human immunodeficiency virus strain cytopathic to megakaryocytic cells. *Blood* 1993; 81: 3336–3342.
43 Riviere C, Subra F, Cohen-Sohlal K et al. Phenotypic and functional evidence for the expression of CXCR4 receptor during megakaryocytopoiesis. *Blood* 1999; 93: 1511–1523.
44 Bussel JB, Haimi JS. Isolated thrombocytopenia in patients infected with HIV: Treatment with intravenous gammaglobulin. *Am J Hematol* 1988; 28: 79–84.
45 Brown SA, Majumdar G, Harrington C et al. Effect of splenectomy on HIV-related thrombocytopenia and progression in HIV infection in patients with severe haemophilia. *Blood Coagul Fibrinolysis* 1994; 5: 393–397.
46 Stricker RB, Shuman MA. Aplastic anaemia complicating systemic lupus erythmatosus: response to androgens in two patients. *Am J Hematol* 1984; 17: 193–201.
47 Debusscher L, Bitar N, DeMaubeuge J et al. Eosinophilic fasciitis and severe aplastic anemia: favourable response to either antithymocyte globulin or cyclosporin A in blood and skin disorders. *Transpl Proc* 1988; 20: 310–313.
48 Suda T, Omine M, Tsuchiya J, Maekawa T. Prognostic aspects of aplastic anemia in pregnancy. Experience on six cases and review of the literature. *Blut* 1978; 36: 285–298.
49 Bacigalupo A, Brand R, Oneto R. Treatment of acquired severe aplastic anemia: bone marrow transplantation compared with immunosuppressive therapy – The European Group for Blood and Bone Marrow Transplantation experience. *Sem Hematol* 2000; 37: 69–80.
50 Horowitz MM. Current status of allogeneic bone marrow transplantation in acquired aplastic anemia. *Semin Hematol* 2000; 37: 30–42.
51 Rosenfeld SJ, Kimball J, Vining D, Young NS. Intensive immunosuppression with antithymocyte globulin and cyclosporine as treatment for severe acquired aplastic anaemia. *Blood* 1995; 85: 3058–3065.
52 Hoffman R, Bruno E, Elwell J, Gewirtz AM, Dekker P. Acquired amegakaryocytic thrombocytopenic purpura: a syndrome of diverse etiologies. *Blood* 1982; 60: 1173–1178.
53 Gewirtz AM, Sacchetti MK, Bien R, Barry WE. Cell-mediated suppression of megakaryocytic thrombocytopenic purpura. *Blood* 1986; 68: 619–626.
54 Benedetti F, de Sabata D, Perona G. T suppressor activated lymphocytes (CD8+/DR+) inhibit megakaryocyte progenitor cell differentiation in a case of acquired amegakaryocytic thrombocytopenic purpura. *Stem Cells* 1994; 12: 205–213.
55 Katai M, Aizawa T, Ohara N et al. Acquired amegakaryocytic thrombocytopenic purpura with humoral inhibitory factor for megakaryocyte colony formation. *Intern Med* 1994; 33: 147–149.
56 King JA, Elkhalifa MY, Lataour LF. Rapid progression of acquired amegakaryocytic thrombocytopenic purpura to aplastic anaemia. *South Med J* 1997; 90: 91–94.
57 Geissler D, Thaler J, Konwalinka G, Peschel C. Progressive preleukaemia presenting amegakaryocytic thrombocytopenic purpura: association of the 5q- syndrome decreased megakaryocytic colony formation and a defective production of Meg-CSF. *Leuk Res* 1987; 11: 731–737.
58 Peng CT, Kao LY, Tsai CH. Successful treatment with cyclosporin A in a child with acquired pure amegakaryocytic thrombocytopenic purpura. *Acta Paediatr* 1994; 83: 1222–1224.
59 El Saghir NS, Geltman RL. Treatment of acquired amegakaryocytic thrombocytopenic purpura with cyclophosphamide. *Am J Med* 1986; 81: 139–142.
60 Young NS, Abkowitz JL, Luzzatto L. New insights into the pathophysiology of acquired cytopenias. In Schechter GP, Berliner N, Telen MJ, eds *Hematology 2000* Washington DC: American Society of Hematology, 2000: 18–38.
61 Rosse WF. Paroxysmal nocturnal haemoglobinuria in aplastic anaemia. *Clin Haematol* 1978; 7: 541–553.
62 Luzzatto L, Nafa K. Genetics of PNH. In Young NS, Moss J, eds. *Paroxysmal nocturnal hemaglobulinuria and the GPI-linked proteins.* New York: Academic Press, 2000; 21–47.
63 D' Andrea AD, Grompe M. Molecular biology of Fanconi anaemia: implications for diagnosis and therapy. *Blood* 1997; 90: 1725–1736.
64 Alter BP. Fanconi's anaemia and malignancies. *Am J Hematol* 1996; 53: 99–110.
65 Strathdee CA, Gavish H, Shannon WR, Buchwald M. Cloning of cDNAs for Fanconi's anaemia by functional complementation. *Nature* 1992; 356: 763–767.
66 Lo Ten Foe JR, Rooimans MA, Bosnoyam-Collins L et al. Expression cloning of a cDNA for the major Fanconi's anaemia gene, FAA. *Nat Genet* 1996; 14: 320–332.

Cambridge Books Online © Cambridge University Press, 2009

67 Auerbach AD. Fanconi anaemia diagnosis and the diepoxybutane (DEB) test. *Exp Hematol* 1993; 21: 731–733.
68 Kaiser TN, Lojewski A, Dougherty C, JuergensL, Sahar E, Latt SA. Flow cytometric characterization of the response of Fanconi's anaemia cells to mitomycin C treatment. *Cytometry* 1982; 2: 291–297.
69 Gluckman E, Auerbach AD, Horowitz MM, Sobocinski KA, Ash RC, Bortin MM et al. Bone marrow transplantation for Fanconi anaemia. *Blood* 1995; 86: 2856–2862.
70 Babior BM. Erythrocyte disorders: anaemias related to disturbance of DNA synthesis (megaloblastic anaemia). In Williams WJ, Beutler E, Erslev AJ, Lichtman MA, eds *Hematology*. 4th edn New York: Mcgraw-Hill; 1990.
71 Wickremasinghe RG, Hoffbrand AV. Reduced rate of DNA replication fork movement in megaloblastic anaemia. *J Clin Invest* 1980; 65: 26–36.
72 Epstein RD. Cells of the megakaryocyte series in pernicious anemia. *Am J Med* 1958; 25: 198.
73 Lindenbaum L, Nath BJ. Megaloblastic anaemia and neutrophil hypersegmentation. *Br J Haematol* 1980; 44: 511–513.
74 Levine PH. A qualitative platelet defect in severe vitamin B12 deficiency. *Ann Intern Med* 1973; 178: 533–539.
75 Gough KR, Read AE, McCarthy CF, Waters AH. Megaloblastic anaemia due to dietary deficiency of folic acid. *Q J Med* 1963; 32: 243.
76 Hoffbrand AV, Newcome BFA, Mollon DL. Method of assay of red cell folate activity and the value of the assay as a test for folate deficiency. *Clin Pathol* 1966; 19: 17–28.
77 De Aizpurua HJ, Cosgrove LH, Ungar B, Toh BH. Auto antibodies cytotoxic to gastric parietal cells in serum of patients with pernicious anaemia. *N Engl J Med* 1983; 309: 625–629.
78 Goldberg LS, Fudenberg HH. The autoimmune aspect of pernicious anemia. *Am J Med* 1969; 46: 489–94.
79 Horton MA, Oliver RT. HLA phenotype A2, B12 in vitamin B12 neuromyelopathy. *J Neurol Sci* 1977; 32: 79–89.
80 Ungar B, Mathews JD, Tait BD, Cowling DC. HLA-DR patterns in pernicious anaemia. *Br Med J* 1981; 282: 768–770.
81 Heaney ML, Golde DW. Myelodysplasia. *N Engl J Med* 1999; 340: 1649–1660.
82 Ries LAG, Kosary CL, Hankey BF, Miller BA, Clegg LX, Edwards BK, eds. *SEER Cancer Statistics Review, 1973–1996*, National Cancer Institute. NIH Pub. No. 99–2789. Bethesda, MD, 1999.
83 Bennett JM, Catovsky D, Daniel MT et al. Proposals for the classification of the myelodysplastic syndromes. *Br J Haematol* 1982; 51: 189–199.
84 Harris NL, Jaffe ES, Diebold et al. The World Health Organisation classification of the hematologic malignancies report of the clinical advisory committee meeting, Airlie House, Virginia, November 1997. *J Clin Oncol* 1999; 17: 3835–3849.
85 Song W-J, Sullivan MG, Legare RD et al. Haploinsufficiency of CBFA2 (AML1) causes familial thrombocytopenia with propensity to develop acute myelogenous leukemia (FDP/AML). *Nat Genet* 1999; 23: 166–174.
86 Bollag G, Clapp DW, Shih S et al. Loss of NF1 results in activation of the RAS pathway and leads to aberrant growth in hematopoietic cells. *Nat Genet* 1996; 12: 144–148.
87 West RR, Stafford DA, White AD et al. Cytogenetic abnormalities in the myelodysplastic syndromes and occupational or environmental exposure. *Blood* 2000; 95: 2093–2097.
88 Hellstrom-Lindberg E, Willman C, Barrett AJ, Saunthararajah Y. Achievements in understanding and treatment of myelodysplastic syndromes. In Schechter GP, Berliner N, Telen MJ, eds. *Hematology 2000*. Washington DC: American Society of Hematology; 2000: 110–132.
89 Lai JL, Preudhomme C, Zandecki M et al. Myelodysplastic syndromes and acute myeloid leukemia with 17p deletion. An entity characterized by specific dysgranulopoiesis and a high incidence of p53 mutations. *Leukemia* 1998; 12: 887–892.
90 Yokota S, Kiyoi H, Nakao M et al. Internal tandem duplication of the FLT3 gene is preferentially seen in acute myeloid leukemia and myelodysplastic syndrome among various hematologic malignancies. A study of a large series of patients and cell lines. *Leukemia* 1997; 11: 1605–1609.
91 Uchida T, Kinoshita T, Nagai H et al. Hypermethylation of the $p15^{INK4b}$ gene in myelodysplastic syndromes. *Blood* 1997; 90: 1403–1409.
92 Hoefsloot LH, Amelsvoort MP, Broeders LCAM et al. Erythropoietin-induced activation of STAT5 is impaired in the myelodysplastic syndrome. *Blood* 1997; 89: 1690–1700.
93 Sugarawa T, Endo K, Shishido T et al. T-cell mediated inhibition of erythropoiesis in myelodysplastic syndromes. *Am J Hematol* 1992; 41: 304–305.
94 Molldrem JJ, Jiang YZ, Stetler-Stevenson MA et al. Hematologic response in patients with myelodysplastic syndrome to antithymocyte globulin is associated with a loss of lymphocyte-mediated inhibition of CFU-GM and alterations in T-cell receptor V beta profiles. *Br J Haematol* 1998; 102: 1314–1322.
95 Jonasova A, Neuwirtova R, Cermak J et al. Cyclosporin A therapy in hypoplastic MDS patients and certain refractory anaemias without hypoplastic bone marrow. *Br J Haematol* 1998; 100: 304–309.
96 Appelbaum FR, Anderson J. Allogeneic bone marrow transplantation for myelodysplastic syndromes: outcomes analysis according to IPSS score. *Leukemia* 1998; 12: 25–29.
97 Silverman LR, Demakos EP, Peterson B et al. A randomized controlled trial of subcutaneous azacytidine (AZA C) in patients with the myelodysplastic syndrome: a study of the Cancer and Leukemia Group B (CALCB). *Proc Am Soc Clin Oncol* 1998; 17: abstr 14.
98 Hellstrom-Lindberg E, Negrin R, Stern R et al. Erythroid response to treatment with G-CSF plus erythropoietin for the anaemia of patients with myelodysplastic syndromes: proposal for a predictive model. *Br J Haematol* 1997; 99: 344–351.
99 Groopman J, Ellman L. Acute promyelocytic leukaemia. *Am J Hematol* 1979; 7: 395–408.
100 Tallman MS, Andersen JW, Schiffer CA et al. All-trans retinoic

Cambridge Books Online © Cambridge University Press, 2009

acid in acute promyelocytic leukemia. *N Engl J Med* 1997; 337: 1021–1028.

101 Gorin NC, Estey E, Jones RJ, Llevitsky HI, Borrello I, Slavin S. New developments in the therapy of acute myelocytic leukemia. In Schechter GP, Berliner N, Telen MJ, eds. *Hematology 2000* Washington DC: American Society of Hematology; 2000: 69–89.

102 Heim S, Mitelman F. *Cancer cytogenetics* 2nd ed New York: Wiley-Liss; 1995.

103 De Sauvage FJ, Carver-Moore K, Luoh SM et al. Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. *J Exp Med* 1996; 183: 651–656.

104 Ballmaier M, Schulze H, Straup G et al. Thrombocytopoietin in patients with congenital thrombocytopenia and absent radii: elevated serum levels, normal receptor expression, but defective reactivity to thrombopoietin. *Blood* 1997; 90: 612–619.

105 Letestu R, Vitrat N, Masse A et al. Existence of a differentiation blockage at the stage of a megakaryocyte precursor in the thrombocytopenia and absent radii (TAR) syndrome.

106 Van den Oudenrijn S, Bruin M, Folman CC et al. Mutations in the thrombopoietin receptor, Mpl, in children with congenital amegakaryocytic thrombocytopenia. *Br J Haematol* 2000; 110: 441–448.

107 Stockelberg D, Andersson, Bjornsson, Bjork S, Wadenvik H. Plasma thrombopoietin levels in liver cirrhosis and kidney failure. *J Intern Med* 1999; 246: 471–475.

108 Kawasaki T, Takeshita A, Souda K et al. Serum thrombopoietin levels in patients with chronic hepatitis and liver cirrhosis. *Am J Gastroenterol* 1999; 94: 1918–1922.

109 Peck-Radosavljevic M, Wichlas M, Zacherl M et al. Thrombopoietin induces rapid resolution of thrombocytopenia after orthotopic liver transplantation through increased platelet production. *Blood* 2000; 95: 795–801.

110 Wolber EM, Ganschow R, Burdelski M, Jelkmann W. Hepatic thrombopoietin mRNA levels in acute and chronic liver failure of childhood. *Hepatology* 1992; 29: 1739–1742.

111 Goulis J, Chau TN, Jordan S et al. Thrombopoietin concentrations are low in patients with cirrhosis and thrombocytopenia and are restored after orthotopic liver transplantation. *Gut* 1999; 44: 754–758.

112 Vadhan-Raj S, Murray LJ, Bueso-Ramos C et al. Stimulation of megakaryocyte and platelet production by a single dose of recombinant human thrombopoietin in patients with cancer. *Ann Intern Med* 1997; 126: 673–681.

113 Abina MA, Tulliez M, Duffour MT et al. Thrombopoietin (TPO) knockout phenotype induced by cross-reactive antibodies against TPO following injection of mice with recombinant adenovirus encoding human TPO. *J Immunol* 1998; 160: 4481–4489.

114 Dale DC, Nichol JL, Rich DA et al. Chronic thrombocytopenia is induced in dogs by development of cross-reacting antibodies to the Mpl ligand. *Blood* 1997; 90: 3456–3461.

115 Ihara K, Ishii E, Eguchi M et al. Identification of mutations in the c-mpl gene in congenital amegakaryocytic thrombocytopenia. *PNAS* 1999; 96: 3132–3136.

116 Cohen T, Cooney DP. Cyclic thrombocytopenia. Case report and review of literature. *Scand J Haematol* 1974; 12: 9–17.

117 Tomer A, Schreiber AD, McMillan R et al. Menstrual cyclic thrombocytopenia. *Br J Haematol* 1989; 71: 519–524.

118 Nagasawa T, Hesegawa Y, Kamoshita M et al. Megakaryopoiesis in patients with cyclic thrombocytopenia. *Br J Haematol* 1995; 91: 185–190.

119 Menitove JE, Pereira J, Hoffman R, Anderson T, Fried W, Aster RH. Cyclic thrombocytopenia of apparent autoimmune etiology. *Blood* 1989; 73: 1561–1569.

Cambridge Books Online © Cambridge University Press, 2009

36

Immune-mediated thrombocytopenia

Kathryn E. Webert and John G. Kelton

Department of Medicine, Faculty of Health Sciences, McMaster University, Hamilton, Ontario, Canada

Immune-mediated thrombocytopenia

Immune-mediated thrombocytopenia is the term used to describe the group of thrombocytopenic disorders in which platelets are destroyed by immune mechanisms. The thrombocytopenia develops when the rate of platelet destruction is greater than the ability of the megakaryocytes in the bone marrow to compensate with increased platelet production. Different investigators have attempted to quantitate this rate of platelet destruction; however, the estimates are relatively imprecise. Many investigators feel that the marrow can compensate at least fivefold without a detectable fall in the platelet count. The platelet destruction in immune-mediated thrombocytopenia can be caused by autoantibodies, alloantibodies, and immune complexes. These are illustrated schematically in Fig. 36.1. Most episodes of immune-mediated platelet destruction are caused by the binding of IgG antibodies to platelet-specific membrane components.

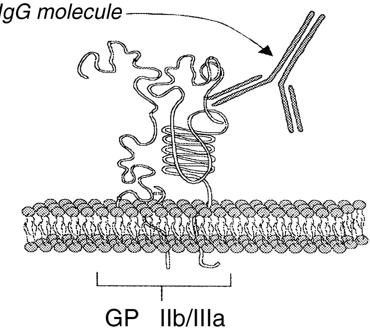
General approach to a patient with suspected immune-mediated thrombocytopenia

When a patient presents with suspected immune-mediated thrombocytopenia, the physician must simultaneously confirm that the patient is thrombocytopenic, begin to determine the general mechanism responsible for the thrombocytopenia, and determine what, if any, treatment is required. Although these various aspects of thrombocytopenia are covered elsewhere in this book, it is appropriate to briefly address each question.

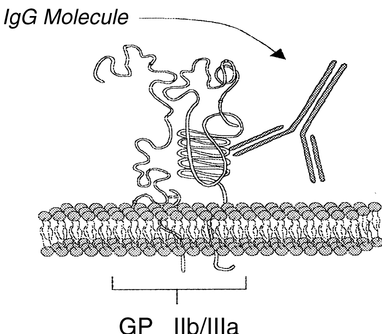
Is the patient thrombocytopenic?

Today, most platelet counts are performed using automated particle counters. With cutbacks in budgets for health care, it has become far less common for every blood film of suspected thrombocytopenic patients to be examined, and even less common for manual (phase-contrast) platelet counts to be performed. Consequently, there is the risk of a patient who has an artefactually low platelet count, also known as pseudothrombocytopenia, to undergo further investigations and even be treated. Pseudothrombocytopenia occurs in 1 in every 500 to 1000 blood samples and occurs when the standard anticoagulant, ethylenediaminetetraacetic acid (EDTA), causes conformational changes in the platelet membrane which cause autoantibodies, typically IgM, to clump the platelets. Automated determination of the platelet count will be inaccurate, as the particle counter will not recognize the larger platelet aggregates as platelets. Pseudothrombocytopenia can have a number of different causes, but is usually due to EDTA-dependent platelet agglutinins. Other causes include cold-dependent platelet clumping, temperature and anticoagulant-independent platelet clumping, and platelet satellitism around leukocytes. All of these artefacts of the platelet count can be detected by examining the stained peripheral blood film. Although an actual platelet count can be difficult to determine, we have found that the most accurate count is obtained by performing a phase-contrast count on a finger prick sample. In summary, the first step when a patient is identified as having thrombocytopenia, particularly if it is asymptomatic thrombocytopenia, is to confirm that the patient is actually thrombocytopenic by examination of the stained blood film.

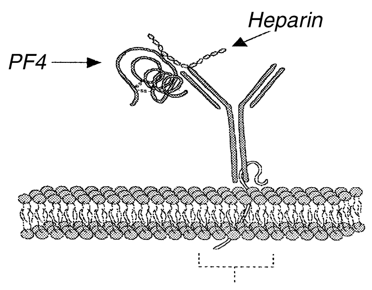
Alloantibodies



Autoantibodies



Drug-dependent antibodies



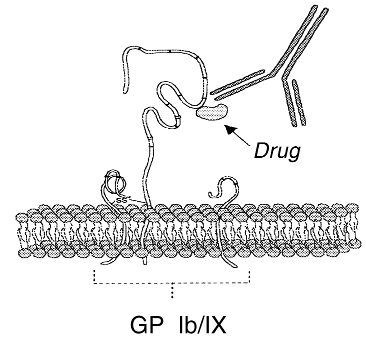


Fig. 36.1. A schematic representation of how different autoantibodies are able to bind to platelets. Most episodes of immune-mediated platelet destruction are caused by the binding of IgG antibodies to platelet-specific membrane components.

Does this patient have immune thrombocytopenia?

This is always the most difficult question of these three questions. Currently, most physicians believe that immune thrombocytopenia is a diagnosis of exclusion. However, recently developed tests to confirm the diagnosis of immune thrombocytopenia (described subsequently) have been shown to have a sufficiently high sensitivity and specificity to prove useful. Thrombocytopenia, like all cytopenias, can be divided into disorders of increased destruction, disorders of underproduction, and disorders of sequestration within an enlarged spleen. Thrombocytopenia caused by increased sequestration in the spleen should be suspected when one documents an enlarged spleen on physical examination, or by videographic confirmation, typically abdominal ultrasound. Hypersplenic thrombocytopenia is confirmed by the performance of autologous platelet survival studies, which document a dramatically reduced platelet recovery (frequently less than 20%), yet a normal or near normal platelet survival. Because these tests are not available to most practising hematologists, the diagnosis of thrombocytopenia caused by splenic sequestration is

Cambridge Books Online © Cambridge University Press, 2009

usually made by confirming mild to moderate thrombocytopenia in association with splenomegaly. Finally, thrombocytopenia caused by reduced production of platelets usually is obvious because of a parallel reduction in the other circulating blood cells, especially the neutrophils, and to a lesser extent the hemoglobin. Examination of the bone marrow helps confirm this diagnosis. Rarely, isolated thrombocytopenia can occur as a consequence of platelet underproduction as seen in amegakaryocytic thrombocytopenia (Chapter 35).

Once the physician has ruled out underproduction and platelet sequestration as a cause of the thrombocytopenia, the mechanism, by default, is an increased rate of platelet destruction. In this category, immune mechanisms predominate. Other causes of increased platelet destruction which can include disseminated intravascular coagulation and generalized infections by viruses, bacteria, or parasites, among other causes, are almost always obvious and usually can be confirmed or excluded by a physical exam in conjunction with appropriate laboratory testing.

In summary, immune thrombocytopenia remains a diagnosis that is made by exclusion; however, there are typical signs and symptoms that can allow a physician to make this diagnosis with considerable confidence. First, unless the patient has a secondary cause for their immune thrombocytopenia, the patient is asymptomatic and has no physical signs with the exception of abnormalities caused by hemostatic impairment. Secondly, many patients with immune thrombocytopenia, especially those with idiopathic thrombocytopenic purpura, have moderate thrombocytopenia with platelet counts ranging from 30 to 100×10^9/l. Most frequently, these patients are entirely asymptomatic and their isolated thrombocytopenia is discovered during routine laboratory testing or after the patient has bled excessively from an otherwise relatively minor hemostatic insult such as surgery or a dental extraction. For many of these patients, a review of previous medical records will confirm that they have had thrombocytopenia for some time that was not detected.

Does the patient with immune thrombocytopenia require treatment?

Recent advances in our understanding of the natural history of immune thrombocytopenia, especially idiopathic thrombocytopenic purpura (ITP), has significantly changed the approach that most physicians use to make management decisions. We now know that many patients with ITP with moderate to severe thrombocytopenia will live healthy, uncomplicated lives and not require any therapy. Many investigators have raised the question of whether the treatment of mild to moderate thrombocytopenia (platelet counts greater than 30×10^9/l) results in greater morbidity than untreated ITP. Our approach is to consider the patient's signs of thrombocytopenia and relate these to the platelet count. For example, patients with mild to moderate thrombocytopenia are almost always asymptomatic and will not have spontaneous bleeding unless they have taken a drug which interferes with platelet function such as aspirin or alcohol. These patients tend to remain asymptomatic. The most frequent question concerning intervention is whether they need treatment to raise the platelet count in anticipation of a surgical procedure such as a tooth extraction or minor surgery. Our general practice is to raise the platelet count if they have moderate thrombocytopenia (platelet count ranging from 30 to 50×10^9/l), with no treatment for those patients whose platelet count is greater than 50×10^9/l. Patients with more severe thrombocytopenia, whose platelet count is less than 20×10^9/l, are more difficult to manage and often require therapy. It is our experience that at a platelet count ranging from 20 to 30×10^9/l, the decision-making should be individualized. Most patients with immune thrombocytopenia whose platelet count is above 30×10^9/l will not require therapy. Conversely, many, but not all, patients whose platelet count is less than 20×10^9/l will require therapy.

There is a hierarchy of symptoms that predict the need for urgent intervention. For example, some patients with platelet counts less than 10×10^9/l are asymptomatic. We tend to treat them, but one may argue that not treating these patients is equally as appropriate. The presence of petechiae and bruising indicates a fairly urgent need for treatment. Furthermore, generalized petechiae suggest that more immediate intervention is required. Blood blisters in the mucous membranes, especially along the bite margins of the buccal mucosa, are an indication of severe hemostatic impairment and the need for urgent intervention. Finally, patients with immune thrombocytopenia can have near fatal hemorrhages, most frequently into the brain or less commonly into the gastrointestinal tract. Almost always, these patients will have had significant bleeding, especially mucous membrane bleeding for some time, which can alert the physician about the need for urgent intervention. The rate of fatal hemorrhages in patients with platelet counts less than 30 X 10^9/l has been estimated to be between 0.012 and 0.130 cases per patient year[1]. For these patients, aggressive therapy including reticuloendothelial (RE) blockade, high-dose corticosteroid therapy and platelet transfusions is indicated.

Cambridge Books Online © Cambridge University Press, 2009

Table 36.1. Immune thrombocytopenia

Type of antibody	Classification
Autoantibody	Primary
	Idiopathic thrombocytopenic purpura
	Acute
	Chronic
	Secondary
	Systemic lupus erythematosus
	Rheumatoid arthritis
	Sarcoidosis
	Lymphoproliferative disorders
Alloantibody	Alloimmune neonatal thrombocytopenia
	Post-transfusional purpura
	Refractoriness to platelet transfusions
Drug-dependent antibody	Quinine, quinidine
	Heparin-induced thrombocytopenia

Autoimmune thrombocytopenia

In autoimmune thrombocytopenia, the immune destruction is caused by autoantibodies. Most of these autoantibodies bind to individual platelet glycoproteins, especially the glycoprotein IIb–IIIa complex (GP IIb–IIIa). These antibodies, which are almost always IgG, can be associated with a number of clinical disorders. Primary immune thrombocytopenia, which is also known as idiopathic thrombocytopenic purpura (ITP), has no underlying disease associations. Secondary immune thrombocytopenia includes a group of diseases (see Table 36.1) which by definition are associated with a systemic disease.

Primary autoimmune thrombocytopenia, idiopathic thrombocytopenic purpura

ITP is the most common cause of autoimmune thrombocytopenia. ITP occurs in both children and adults, and, simplistically, the childhood form can be considered to be the 'inverse' of the adult form. Eighty per cent of children with ITP will have an acute illness, defined as less than 6 months of disease with spontaneous remission of their illness. Only 20% of children with ITP will have chronic disease requiring ongoing therapy. In contrast, 80% of adults presenting with acute ITP will prove to have chronic ITP. Many will require more definitive treatment such as splenectomy. Although childhood ITP is described elsewhere in this book (Chapter 37), we will briefly summarize its key aspects. Childhood ITP is equally frequent in boys and girls and typically presents in children less than 5 years of age. Often, the children will have had an antecedent infection or, less commonly, a vaccination. The ITP is often dramatic in its presentation as the thrombocytopenia can be quite severe, with platelet counts falling to less than 10×10^9/l. Most children are symptomatic and have evidence of hemostatic impairment with petechiae and purpura. Untreated, the illness usually resolves spontaneously. Although catastrophic hemorrhage such as an intracranial hemorrhage is very rare in these children (less than one percent), many physicians would treat patients who have severe thrombocytopenia and signs of hemostatic impairment with interventions such as high-dose intravenous immunoglobulins (IVIg) or high-dose corticosteroids to speed up their recovery. The extent of the investigation and the necessity for intervention is debated and is described in greater detail in Chapter 37. The likelihood of having chronic disease and requiring ongoing intervention or splenectomy increases with age and rises dramatically to adult levels following puberty. Although there is no sex predominance in the young child with ITP, girls are more frequently affected when ITP presents in adolescence or adulthood.

Adults with ITP may present in one of three ways. About one-third of patients who ultimately are diagnosed as having ITP are asymptomatic, and their thrombocytopenia is discovered at the time of routine blood testing. These patients typically have a platelet count greater than 50×10^9/l and they have mild idiopathic thrombocytopenic purpura. About one-third of patients will give a history of intermittent episodes of bruising that have occurred for months to years. Sometimes, the patient will note that the bruising was associated with alcohol consumption or the use of an antiplatelet agent such as aspirin. This group of patients typically has moderate thrombocytopenia and a platelet count ranging from 20 to 50×10^9/l. Finally, about one-third of ITP patients will present with fairly dramatic and abrupt evidence of hemostatic impairment with petechiae, purpura, and, less commonly, mucous membrane bleeding. These patients often have a platelet count that is less than 20×10^9/l. Treatment is required for these patients.

Physical and laboratory investigations in patients with ITP

The physical examination of ITP patients should parallel the approach described earlier in this chapter. The physician should simultaneously evaluate whether the patient has evidence of secondary immune thrombocytopenia and determine the degree of hemostatic impairment.

Cambridge Books Online © Cambridge University Press, 2009

Patients should be carefully examined for evidence of petechiae. Petechiae are rare at platelet counts greater than 20×10^9/l unless there is a platelet function disorder. Petechiae are tiny (1 to 3 mm) collections of red blood cells that have leaked from capillaries. Petechiae are quite specific for thrombocytopenia and should be differentiated from angioma, which are more commonly found on the upper limbs and abdomen than on the feet and lower limbs. Ecchymoses or bruises are much less specific for thrombocytopenia but also provide useful information since they can help alert the physician about the chronicity of the ITP. The rest of the physical examination should focus on looking for secondary evidence of immune thrombocytopenia such as lymphadenopathy, splenomegaly and hepatomegaly.

The laboratory investigations include an accurate platelet count with examination of the peripheral blood film to exclude pseudothrombocytopenia. Other investigations include evaluation of thyroid function because of the strong association of thyroid hypo- and hyper-function with ITP. Antinuclear antibody serology, antiphospholipid antibodies, and anticardiolipin antibodies are performed if there is suspicion of an associated immune disorder. These tests should also be performed if the female patient has had a history of pregnancy losses suggesting the antiphospholipid antibody syndrome. The performance of other investigations, including viral serology (Epstein–Barr virus and HIV serology, etc.), would depend upon the presentation and associated clinical findings.

We would not perform a bone marrow in a patient with typical ITP. However, one should perform a bone marrow aspiration and examination if the patient has abnormalities seen on examination of the stained blood film or when there are multiple blood cell lineages affected. For example, we have identified elderly patients who appeared to have immune thrombocytopenia, but who proved to have a myelodysplastic syndrome. Often, the thrombocytopenia in these patients is accompanied by macrocytosis. Additionally, the diagnosis of ITP should be questioned if the patient has a poor response to standard therapy. The examination of the bone marrow should include special stains plus cytogenetic analysis, since chromosome abnormalities can found in myelodysplastic syndromes. The finding of normal numbers or increased numbers of megakaryocytes in the bone marrow is consistent with the diagnosis of ITP. Although some investigators have described abnormalities of megakaryocytes including multinuclei, we have not found this to be helpful in the discrimination of ITP from other thrombocytopenic disorders.

Although not currently recommended for the diagnosis and evaluation of ITP[2], various other tests are currently being investigated to diagnose and characterize patients with ITP. These tests include the measurement of reticulated platelets[3–6], serum thrombopoietin levels[6–9] and measurement of platelet glycocalicin[6,7,10].

Platelet antibody testing

A variety of tests have been developed to detect platelet-associated antibodies[11,12]. These assays have been classified into three groups termed phase I, II, and III assays according to the time of introduction and to the general technique involved. Initially, in the 1950s, antiplatelet antibodies were detected by phase I assays. These assays involved the mixing of the patient's serum with control platelets from healthy volunteers. Various platelet-dependent endpoints are measured including platelet aggregation, platelet release, platelet lysis and platelet procoagulant formation. These assays have a low sensitivity and specificity for ITP and, therefore, are no longer used for its diagnosis.

Phase II assays detect platelet-associated IgG (PAIgG) by measuring both specific and non-specifically bound IgG (as well as complement and other immunoglobulins) on the platelet surface. Phase II assays are generally superior to phase I assays because they directly detect IgG or complement on the platelet, rather than infer its presence through an indirect endpoint such as platelet activation. The PAIgG is typically measured by one of three methods: two-stage methods, direct binding assays, and total PAIgG assays[12,13]. In the two-stage method, a known amount of anti-immunoglobulin is incubated with washed platelets. The residual anti-immunoglobulin is then measured and the amount of IgG on the platelets is calculated. Direct binding assays use labelled polyclonal or monoclonal anti-human antibodies to directly quantify the amount of IgG bound to platelets. These assays are performed by incubating the washed test platelets with labelled anti-Ig probe. The amount of labelled probe bound to the platelets is then measured. Finally, total PAIgG assays measure the total PAIgG concentration found on the surface and within the platelet. The patient's platelets are lysed which releases IgG from the membrane, cytosol, and alpha-granules. The immunoglobulin can be measured by a variety of techniques including radioimmunoprecipitation and nephelometry. In patients with ITP, the PAIgG is usually elevated, however, the diagnostic usefulness of this test is limited. These assays produce positive results for most thrombocytopenic patients. However, the finding of elevated PAIgG is not specific for or diagnostic of ITP, as levels are also ele-

Cambridge Books Online © Cambridge University Press, 2009

vated in patients with other non-immune-mediated thrombocytopenic conditions[14,15]. In a study by Kelton et al. the sensitivity of the PAIgG test for clinically diagnosed ITP was found to be 91% and the specificity was 27%. The positive predictive value of an elevated PAIgG as a diagnostic test of ITP in a thrombocytopenic patient was only 49%, while the negative predictive value was 82%[15].

Phase III assays measure the binding of antiplatelet autoantibodies to specific platelet glycoproteins. These assays detect and measure platelet glycoprotein-specific antibodies that recognize GP IIb/IIIa and GP Ib/IX. By measuring antibodies binding to specific glycoproteins, these assays overcome the problem of non-specific binding of IgG that complicates phase II assays. The phase III assays include immunoblotting, radioimmunoprecipitation, and antigen capture or glycoprotein immobilization assays[11]. In immunoblotting or Western blotting, the patient's serum is added to platelet proteins that have been separated by electrophoresis and immobilized on nitrocellulose. The binding of a patient antibody to specific protein bands is detected using a labelled anti-Ig probe. In radioimmunoprecipitation assays, the patient's serum is added to radiolabelled platelet membrane proteins. The protein–antibody complexes are precipitated by the addition of an anti-Ig bound to a solid phase. The precipitated platelet proteins are then separated by sodium dodecylsulfate polyacrilamide gel electrophoresis and detected by autoradiography. Antigen capture assays use monoclonal antibodies to capture specific platelet proteins (for example, GP IIb/IIIa). Labelled anti-Ig is used to detect the patient's IgG bound to captured platelet proteins. Large prospective studies have demonstrated phase III assays to have a high specificity for the diagnosis of ITP but to be relatively insensitive[16,17]. In a prospective study, Warner et al. demonstrated the specificity of these assays to be 92% and the sensitivity to be from 39 to 66%[17].

Treatment of idiopathic thrombocytopenic purpura

The most important decision to make is if the patient requires any treatment. Mild to moderate thrombocytopenia in an asymptomatic patient who will not be undergoing hemostatic challenges such as elective surgery, often does not require any treatment. Again, and as noted previously, we would typically not treat patients whose platelet count was above 30×10^9/l and often will treat a patient whose platelet count was less than 20×10^9/l. For asymptomatic patients with mild to moderate thrombocytopenia (platelet count greater than 30×10^9/l), we organize appropriate serological tests and then monitor the platelets, initially on a weekly, then on an every-other-week basis for 3 to 6 months. The patient maintains a symptom diary and also records his or her own platelet count. Typically, patients with mild to moderate thrombocytopenia will have a consistent platelet count over the initial interval of monitoring. We gradually increase the interval of the platelet monitoring to once a month. We have monitored many of these patients and we have found that they tend to maintain a consistent platelet count that decreases only if the patient has had an immune stimulus, such as an infection. The majority of these patients will never be treated, but they are made aware that it may be necessary to raise the platelet count before a hemostatic challenge such as a tooth extraction. For patients with mild to moderate thrombocytopenia undergoing less extensive surgery, we would often treat them with a fibrinolytic inhibitor such as epsilon aminocaproic acid. Platelet transfusions are almost never required.

Patients with severe thrombocytopenia, and platelet counts less than 10×10^9/l, almost always require treatment, particularly if they have evidence of hemostatic impairment. The first line of treatment is corticosteroids or RE blockade. The corticosteroids are typically given as oral prednisone at a dose of one milligram per kilogram (rounded off to the nearest 5 to 10 milligrams); alternatively, RE blockade using Rho (D) immunoglobulin (anti-D) in an Rh positive individual (75 μg/kg intravenously), or high-dose intravenous immunoglobulins (IVIg) at a dose of 1 to 2 grams per kilogram. Both corticosteroids and RE blockade are equally effective and will raise the platelet count in about two-thirds of patients. Corticosteroids are more likely to produce side effects including Cushing's disease, hypertension, diabetes mellitus, and osteoporosis. RE blockade has fewer side effects, but a higher cost. When corticosteroids are used, they should be given for as short an interval as possible and a taper begun as soon as the platelet count has reached hemostatically safe levels, such as greater than 100×10^9/l. During the tapering phase, we will change the patient to alternate day corticosteroids, since this is more likely to have fewer adverse side effects with the same immunosuppressive effects. RE blockade using anti-D or high-dose IVIg causes the platelet count to rise within days, peak within one to two weeks, and then slowly fall over the next four to eight weeks. About 10 to 20% of adult patients who present with apparent acute ITP (no history of bruising prior to presentation) will have a complete recovery of their platelet count or will achieve a safe platelet count off all therapy. The remaining 80% of patients require more definitive therapy, and recognizing this fact, we proceed to splenectomy early in the treatment.

Cambridge Books Online © Cambridge University Press, 2009

Splenectomy

Splenectomy is the definitive therapy for ITP, and we perform this procedure within six months of the first presentation of ITP in adults if the patient requires ongoing therapy. Prior to splenectomy, the patient should receive pneumococcal, meningococcal, and probably *Hemophilus influenza* vaccines. The vaccine should precede the splenectomy by at least two weeks. Patients are prepared for surgery by raising their platelet count before the procedure. Again, platelet transfusions are rarely required, even in those patients with very severe thrombocytopenia who require surgery. Because of its reduced morbidity and significantly shortened hospital stay, laparoscopic splenectomy is preferred over open splenectomy[18]. Splenectomy will result in a long-term remission or cure in more than 70% of patients[19]. In general, patients who do not have a normal platelet count following splenectomy are easier to manage.

Patients who have a complete remission following splenectomy have less than 20% risk of relapse. Patients who do relapse, particularly after a prolonged interval of normal platelet count, should be investigated for the presence of an accessory spleen. Studies using sensitive imaging techniques have shown that as many as 40% of these patients will have residual splenic tissue[20–22]. It is important to recognize that the presence of postsplenectomy changes in the peripheral blood film do not rule out the possibility of an accessory spleen[20,22]. It has been estimated that about half of patients will have a second remission after removal of an accessory spleen, although our personal experience has been less positive[20–22].

Management of a patient with chronic refractory ITP

About one-third of patients with ITP who require therapy will fail splenectomy and have platelet counts less than 20×10^9/l. The optimal approach to these patients remains uncertain. Issues such as age and anticipated natural lifespan, mobility of the platelet count, and, finally, tolerance to medication all must be considered when discussing future therapy. For these patients, we believe that it is particularly important to have a full discussion of treatment options including the risks and benefits. For example, some patients with severe ITP and platelet counts less than 10×10^9/l will not have significant hemostatic impairment, and these patients can be monitored with consideration of short-term interventions such as high-dose IVIg or corticosteroids at the time of hemostatic challenge such as surgery.

A significant proportion of refractory patients require therapy, and we use a 'staircase' approach with the progressive addition of more medicines.

Some patients can be maintained at a safe platelet count using intermittent corticosteroids delivered every few days to every few weeks or high-dose IVIg given every few weeks to every few months. For this type of patient, we arrange outpatient blood testing and recommend that the patient maintain a diary of symptoms as well as record their own platelet count. Sometimes, patients can be managed with sufficiently low doses of corticosteroids given at sufficiently long intervals that long-term side effects are minimized. If larger doses of corticosteroids are used, steps should be taken to prevent osteoporosis.

The next 'step in the staircase' is the addition of the anabolic steroid danazol, which can be delivered at doses of 200 to 1200 milligrams per day. This drug causes dose-dependent elevation of liver enzymes and can cause hepatotoxicity. Therefore, its use requires careful monitoring and seldom can patients be maintained at high doses for long intervals. Vinca alkaloids, such as vincristine and vinblastine occasionally can cause a short-term rise in the platelet count, but treatment is usually limited by dose-dependent neurotoxicity. These medications are usually administered intravenously at weekly intervals. Some investigators have reported remissions induced by the administration of platelets that have been incubated with either vincristine or vinblastine[23,24]. However, equivalent responses have been obtained by simply infusing these medications[25].

Refractory patients who do not respond to any of these manoeuvres have about a 50% likelihood of responding to any of the following agents, which we typically use in combination: cytotoxic agents, such as cyclophosphamide or azathioprine, and broad immunosuppressant agents such as cyclosporine. For both of these classes of agents, we administer them orally and at moderate doses over long intervals. Typically, an interval of 3 to 6 months is required to know whether a patient will respond to therapy. It is our opinion that some of the most common errors in managing patients with severe and refractory ITP is the ongoing use of potent agents which produce side effects (corticosteroids, vinca alkaloids, cytotoxic agents, etc.), when the patient has had no response. Sometimes, we see patients who have had greater morbidity from the treatment than from nontreated disease.

Emergency treatment of ITP

Patients with ITP who have acute or potentially catastrophic bleeding require aggressive therapy including platelet transfusions, high dose IVIg, and high-dose corticosteroids, in addition to standard resuscitation that can include blood replacement if required.

Cambridge Books Online © Cambridge University Press, 2009

Experimental treatments of ITP

A large number of new therapies for ITP are currently being investigated. Emilia et al., in a small number of patients, have demonstrated that eradication of *Helicobacter pylori* infection led to a complete response in 33% of patients treated and a partial response in 17% of patients[26]. Other agents being investigated for the treatment of refractory ITP include rituximab, an anti-CD20 monoclonal antibody[27,28], rhuIL-11[29], MDX-33, a monoclonal antibody directed against the Fcγ1 receptor[30], taxol[31] and immunoabsorption over a protein-A sepharose column[32,33].

ITP in pregnancy

Because ITP is a common illness that affects young women, physicians often manage pregnant patients with ITP. In this area, there has been a dramatic evolution in our understanding of the natural history, the outcome for both mother and infant, and consequently the requirement for intervention. We remind pregnant patients with ITP that their overall morbidity and the risk to their infant is very small. Nonetheless, they will require frequent physician visits during the pregnancy with careful monitoring of both mother and infant at the time of delivery. In the past, physicians tended to assume that ITP was the usual cause of thrombocytopenia in pregnancy. We now recognize that immune disorders of pregnancy are uncommon and contribute overall to less than five percent of all episodes of thrombocytopenia in pregnancy. Large prospective studies have shown that about 74% of thrombocytopenias in pregnancy are caused by incidental thrombocytopenia of pregnancy; 21% are caused by hypertensive disorders of pregnancy; and 4 percent are caused by immune disorders of pregnancy[34] (Fig. 36.2).

Incidental thrombocytopenia of pregnancy is defined as mild thrombocytopenia (platelet count of 80 to 150×10^9/l) that occurs at the time of delivery in an otherwise well woman. This is not a disorder, nor does it have implications for mother or her infant. By investigating a large cohort of these women, we have found that these women can safely have epidural analgesia at the time of delivery, and their infants are well and not thrombocytopenic.

Approximately 2 in 1000 births are delivered to women with ITP, and overall ITP accounts for three percent of women with thrombocytopenia in pregnancy[35]. A series of prospective and retrospective studies have clarified the risk to the infant if the mother has ITP. About 10% of infants born to mothers with ITP will have a platelet count less than 50×10^9/l, less than 5% will have a platelet count less than 20×10^9/l, and less than 1% of the infants will have severe thrombocytopenia[36]. ITP remains a challenge for

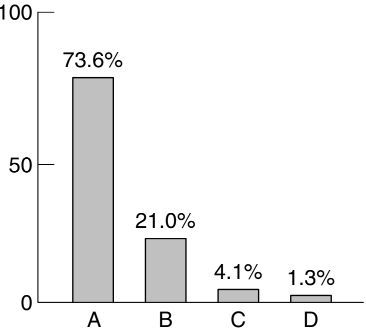


Fig. 36.2. A schematic representation of the overall frequency of the different causes of low platelets in pregnant patients. This figure was constructed from a large prospective study of some 15 000 mother–infant pairs[34].

physicians because there remains a risk, although it is less than 1%, that patients with ITP in pregnancy will have catastrophic outcomes. In utero death due to fetal hemorrhage is described, as is the occurrence of severe thrombocytopenia in the infant after delivery. Many studies have attempted to identify which infant will have thrombocytopenia. There is general agreement that the maternal platelet count and measurement of IgG in maternal plasma cannot be used to predict which infant will be thrombocytopenic[34,37–47]. The best predictor appears to be whether the mother had a previously thrombocytopenic infant[44,46,48,49]. Because of this dilemma, and the fact that some infants born to mothers with ITP will have severe thrombocytopenia, investigators have studied a number of approaches for measuring the infant's platelet count in utero. These have included percutaneous umbilical blood sampling (PUBS) and fetal scalp sampling. We do not favour either approach. Depending upon the centre and their experience, PUBS carries a 2 to 5% procedure-related complication rate[50]. We believe it is difficult to justify any diagnostic manoeuvre with a morbidity or mortality rate that is higher than the illness itself.

Fetal scalp sampling can be performed when the membranes are ruptured and the cervix has begun to dilate. However, our experience and that of others is that this test often produces an erroneously low infant platelet count, which could lead to unnecessary intervention.

During the pregnancy, we monitor the woman's platelet counts. The frequency of platelet count measurement is related to the severity of the maternal thrombocytopenia.

Cambridge Books Online © Cambridge University Press, 2009

Because the platelet count typically worsens later in the pregnancy, we increase the frequency of monitoring to once every one to two weeks in the later part of pregnancy. If intervention is required, we prefer RE blockade using high-dose IVIg over corticosteroids, since corticosteroids have been associated with hypertension in pregnancy. Our approach to the investigation and management of ITP in pregnancy is discussed at length elsewhere[36]. It is important to alert the neonatologists that a woman with ITP will soon deliver. A platelet count should be collected from the cord sample at the time of delivery with future monitoring related to this count. Most infants will have a further fall in their platelet count for several days following delivery, and the majority has the platelet nadir by day two. Treatment includes corticosteroids and high-dose IVIg. Platelet transfusions should be given if bleeding is present or if the thrombocytopenia is particularly severe.

Secondary immune thrombocytopenias

Immune thrombocytopenia can occur in association with a variety of systemic disorders. These diseases are typically disorders of immune dysregulation and include infections, collagen vascular diseases such as systemic lupus erythematosus, lymphoproliferative disorders, and other autoimmune diseases (Table 36.2). Treatment of the thrombocytopenia includes treatment of the underlying disease and therapies as described for ITP. In some conditions, notably Evan's syndrome, the thrombocytopenia is less responsive to therapies.

Table 36.2. Secondary associations of immune thrombocytopenia

Infections
Human immunodeficiency virus
Varicella
Epstein–Barr virus
Collagen vascular disease
Systemic lupus erythematosus
Rheumatoid arthritis
Progressive systemic sclerosis
Sjogren's syndrome
Lymphoproliferative disorders
Chronic lymphocytic leukemia
Hodgkin's disease
Non-Hodgkin's lymphoma
Other
Antiphospholipid antibody syndrome
Autoimmune thyroid dysfunction
Sarcoidosis
Post-bone marrow transplantation
Inflammatory bowel disease
Autoimmune hemolytic anemia (Evan's syndrome)
Bullous pemphigoid
Myasthenia gravis

Human immunodeficiency virus (HIV)-associated ITP

Thrombocytopenia is common, occurring in approximately 20% of patients with symptomatic disease and 9% of asymptomatic seropositive patients[51]. Thrombocytopenia may be the presenting manifestation of HIV infection and the risk of HIV should be assessed in any ITP patient with HIV risk factors. Patients have immune-mediated destruction of their platelets, similar to ITP. Other mechanisms also contribute to the thrombocytopenia including a defect in platelet production due to direct infection of megakaryocytes and sometimes the suppressive effects of medications. Patients with severe thrombocytopenia should be treated similarly to patients with ITP. Most patients respond to steroid therapy, however, only 10 to 20% will have a sustained response[52]. It has been suggested that the use of high-dose dexamethasome may actually be detrimental[53]. IVIg induces a transient improvement in the platelet count, but patients will require repeated treatments[54]. Splenectomy will induce improvement in most patients and there is no evidence that it increases the rate of progression to acquired immunodeficiency syndrome[55]. Treatment with retroviral medications also has a modestly beneficial effect on the thrombocytopenia[55,56]. Interferon-alpha may be a safe and effective treatment of zidovudine-resistant thrombocytopenia[57].

Thrombocytopenia complicating systemic lupus erythematosus

Thrombocytopenia can occur in up to 25% of patients with systemic lupus erythematosus (SLE)[58]. The thrombocytopenia is usually caused by autoantibodies. Some patients will also have concomitant platelet dysfunction characterized by increased bleeding and bruising. The treatment is similar to that for ITP, although the response to splenectomy may be lower with studies showing only 14% of patients not requiring additional treatment after splenectomy[59].

A subset of patients with SLE or SLE-like disorders have antibodies which interfere with phospholipid-dependent coagulation reactions, commonly detected by an unexplained elevation of the patient's prothrombin time (PT) or

Cambridge Books Online © Cambridge University Press, 2009

international normalized ratio (INR). These antibodies are immunoglobulins with specificity for negatively charged phospholipids and are termed as lupus anticoagulant antibodies. They tend to be heterogenous in their epitope specificity with most binding protein complexes including β_2-glycoprotein I[60]. Another class of antibodies, the anticardiolipin antibodies, is detected by an enzyme-linked immunosorbent assay (ELISA) using cardiolipin as the antigen. Cardiolipin is a lipid present in high concentrations in the inner membrane of mitochondria. This is the same antigen that is detected in the VDRL test for syphilis, which explains the false positive VDRL test in these patients. The two classes of antibodies are distinct, but likely have overlapping specificities. In fact, most anticardiolipin antibodies actually recognize an epitope on β_2-glycoprotein I. The term 'antiphospholipid antibodies' applies to both sets of antibodies.

Antiphospholipid antibodies can be associated with both venous and arterial thrombosis. The antiphospholipid antibody syndrome is a syndrome that can include any combination of arterial and venous thrombosis, recurrent fetal losses and thrombocytopenia. In the literature, thrombocytopenia has been found to occur in approximately 20 to 60% of patients with antiphospholipid antibody syndrome[61–63]. The diagnosis should be considered in patients who develop such events as idiopathic deep vein thrombosis, arterial thrombosis, or thrombosis in highly unusual sites. Many of these patients will also have an unusual vascular rash termed livedo reticularis. Patients can have hematologic abnormalities including mild thrombocytopenia, platelet dysfunction and, less commonly, autoimmune hemolytic anemia and leukopenia. As the thrombocytopenia is usually mild, treatment is rarely necessary. Many patients require long-term anticoagulation therapy to prevent recurrent thrombotic events.

Thrombocytopenia secondary to lymphoproliferative disorders

Immune thrombocytopenia commonly complicates chronic lymphocytic leukemia (CLL)[64]. This should be differentiated from thrombocytopenia of underproduction, which is seen in the spent stage of CLL. Immune thrombocytopenia is seen in 1 to 2% of patients with Hodgkin's disease and can predate or postdate the illness[65]. When it occurs after the illness, it does not necessarily indicate a relapse of disease. In some patients, the thrombocytopenia will resolve with treatment of the underlying malignancy. In others, the thrombocytopenia requires specific treatment. These patients should be treated similarly to patients with ITP.

Alloimmune thrombocytopenia

Alloimmune thrombocytopenia is caused by alloantibodies against specific platelet glycoproteins. Alloimmune thrombocytopenic disorders include alloimmune neonatal thrombocytopenia, post-transfusional purpura and refractoriness to platelet transfusions. Alloimmune thrombocytopenia has also been documented after organ transplantation[66].

Alloimmune neonatal thrombocytopenia

Alloimmune neonatal thrombocytopenia is mediated by alloantibodies in maternal plasma directed against paternal alloantigens on fetal platelets[67]. The most common and most important alloantibody to cause this disorder in the Caucasian population is targeted against a platelet glycoprotein termed PL^{A1} (also known as HPA-1a) located on platelet glycoprotein IIIa[68]. The risk of developing neonatal alloimmune thrombocytopenia appears to be associated with maternal HLA antigens, predominantly HLA DRB3*0101 or DQB1*02[69]. This disorder causes severe and often life-threatening fetal thrombocytopenia that can occur in utero. Intracranial hemorrhages occur in 10 to 15% of untreated infants with neonatal alloimmune thrombocytopenia with almost half of these hemorrhages occurring *in utero*[68]. To confirm the diagnosis of neonatal alloimmune thrombocytopenia, serological and genetic testing of both the father and the mother must be performed.

Post-transfusion purpura

Post-transfusion purpura (PTP) is a rare transfusion reaction. The patient, usually a woman, develops severe thrombocytopenia 5 to 12 days after receiving a transfusion of a blood product containing platelets. The thrombocytopenia is often very severe (platelet count less than 10×10^9/l). Post-transfusional purpura occurs when a patient produces an alloantibody to a specific platelet antigen that she lacks. The antigen usually implicated is PL^{A1}. Similarly to neonatal alloimmune thrombocytopenia, the risk for developing PTP is increased in HLA-DR3-positive individuals[70]. The syndrome most commonly occurs in multiparous women because previous pregnancies lead to their sensitization. Rarely, patients (including men) who have previously been transfused are also at risk.

Platelets of both the donor and the patient are destroyed. The destruction of the donor platelets occurs because of the presence of platelet-specific alloantibodies. There is no clear explanation for why the recipient's platelets are also destroyed.

Cambridge Books Online © Cambridge University Press, 2009

The diagnosis of PTP is made by the identification of a platelet-specific antibody in a patient with acute onset of thrombocytopenia five to twelve days after receiving a transfusion of a blood product. Although PTP is most commonly seen after transfusion of packed red blood cells, all blood products, including plasma, can cause the reaction. PTP is self-limited with recovery usually occurring within 1 to 3 weeks. However, because the condition can be lethal, treatment with plasmapheresis or IVIg should be considered. Platelet transfusions should be avoided except in cases of life-threatening hemorrhage.

Refractoriness to platelet transfusions

Alloimmunization is also seen in patients receiving repeated platelet transfusions. In these patients, development of alloantibodies, usually against HLA class 1 antigens, results in the patient being refractory to further platelet transfusions. If alloimmunization is suspected, the patient may have a better increment in platelet count if he is transfused with platelets from a HLA-compatible donor.

Drug-induced thrombocytopenia

Many drugs have been demonstrated to cause thrombocytopenia. The medications most commonly implicated include heparin, quinidine, sulfonamides and gold. However, virtually every medication has been associated with thrombocytopenia and the diagnosis should be considered in any patient who develops unexpected thrombocytopenia.

Patients with drug-induced thrombocytopenia typically have moderate to severe thrombocytopenia and can present with symptoms such as petechiae and mucosal bleeding. The thrombocytopenia is usually seen one to two weeks after beginning a medication, but it may occur unpredictably in patients who have been taking the medication for several years. The platelet destruction is usually IgG-mediated, although it can be mediated by IgM or complement. The thrombocytopenia usually resolves within days of stopping the causative drug. In cases of severe thrombocytopenia, the patient may be treated by reticuloendothelial blockade using either IVIg or intravenous Rho (D) immune globulin if the patient is Rh-positive. Treatment with corticosteroids is less effective. In cases of life-threatening hemorrhage, platelet transfusions may be required. Patients should be instructed to not take the drug causing the thrombocytopenia again as it will cause thrombocytopenia with subsequent exposure. If required, a structurally different drug should be substituted.

Heparin-induced thrombocytopenia

Heparin-induced thrombocytopenia (HIT) is the most important drug-induced thrombocytopenia. Heparin causes thrombocytopenia in 1 to 3% of patients exposed[71]. It usually develops between five and eight days after the initiation of heparin therapy but if the patient has been exposed to heparin within the last 3 months, the thrombocytopenia can occur earlier[72]. Patients develop mild to moderate thrombocytopenia, with platelet counts commonly ranging from 40 to 80×10^9/l. In addition, patients with HIT frequently develop thrombotic complications, especially deep venous thrombosis and pulmonary embolism. Other clinical associations include arterial thrombosis, skin lesions, and uncommon thrombotic events such as adrenal gland thrombosis and hemorrhage.

HIT is caused by an IgG antibody which recognizes a complex of heparin and platelet factor-4 (PF-4)[73,74]. Heparin itself is not immunogenic; rather, the immunogenicity arises from the conformational change in the PF-4 caused by heparin. The PF-4/heparin/IgG immune complexes bind to platelet Fc receptors causing platelet activation and microparticle formation with resulting activation of coagulation[75,76].

The frequency of HIT varies among clinical settings. The risk of thrombocytopenia also appears to be related to the type, dose and duration of heparin administration. For example, unfractionated heparin is more immunogenic than low molecular weight heparin preparations. As well, different patient populations have different risks of forming HIT-IgG. For example, the risk of HIT-IgG is higher in orthopedic patients than in medical patients[77].

The diagnosis of HIT should be considered in all patients receiving heparin therapy who develop thrombocytopenia or the clinical manifestations discussed previously. Other causes of thrombocytopenia should be excluded. Serological tests are used to confirm the diagnosis of HIT. The ELISA-based test was described by Amiral and colleagues[73]. This assay measures the binding of platelet antibodies to a complex of heparin and PF-4. The gold standard tests are biological assays, such as the ^{14}C-serotonin release assay[78]. In these assays, ^{14}C-serotonin labelled platelets from normal donors are incubated with the test serum. The amount of ^{14}C-serotonin released is quantified as a measurement of platelet activation. These assays are more complex and time consuming than the ELISA-based assays. Recent studies have shown that many patients who make the antibodies that cause HIT will not go on to develop the thrombocytopenia or thrombotic complications.

Treatment of HIT involves discontinuation of heparin.

Cambridge Books Online © Cambridge University Press, 2009

The patient should be treated with an agent that inhibits thrombin generation, such as hirudin or argatroban. Warfarin should not be used to treat acute HIT because it can trigger warfarin-induced limb gangrene. In addition to reducing the circulating concentrations of the vitamin K-dependent clotting factors, warfarin also reduces the levels of protein C and protein S, which act as natural anticoagulants.

Gold-induced thrombocytopenia

Gold-induced thrombocytopenia occurs in as many as 3% of patients treated with this medication[79]. There appears to be a genetic predisposition to the syndrome with HLA DR3 occurring in up to eighty percent of affected patients[80]. The thrombocytopenia usually occurs within the twenty weeks of therapy and can range from mild to severe. The initial management of gold-induced thrombocytopenia is to stop the drug and provide supportive treatment if required. Despite discontinuation of the medication, the thrombocytopenia can persist for many months or even years[81]. This is likely due to persistence of an autoantibody, but may also be due to the prolonged release of gold from tissue stores. Rapid correction of the thrombocytopenia may be achieved with IVIg; however, a relapse of the thrombocytopenia may occur in two to four weeks once the effects of the IVIg have worn off. Patients also respond to corticosteroids. Some patients with persistent thrombocytopenia may respond to splenectomy or immunosuppressive medications. Gold-chelating agents such as dimercaprol or N-acetylcysteine may promote gold excretion[82–84].

Quinidine-induced thrombocytopenia

Quinidine or quinine may cause thrombocytopenia in approximately 0.1 to 0.2% of patients, however, it is often difficult to establish a history of drug ingestion because quinidine is present in many tonics and beverages. Patients often present with severe thrombocytopenia and bleeding. In quinine-induced thrombocytopenia, the antibody recognizes a quinine-platelet complex or a drug-induced neoantigen on the platelet surface[85]. The antibody-bound platelets subsequently are rapidly cleared by the Fc receptors of the reticuloendothelial cells. The platelet count usually returns to normal within several days of stopping the medication.

References

1 Cohen YC, Djulbegovic B, Shamai-Lubovitz O, Mozes B. The bleeding risk and natural history of idiopathic thrombocytopenic purpura in patients with persistent low platelet counts. *Arch Intern Med* 2000; 160: 1630–1638.

2 George JN, Woolf SH, Raskob GE. Idiopathic thrombocytopenic purpura: a guideline for diagnosis and management of children and adults. American Society of Hematology. *Ann Med* 1998; 30: 38–44.

3 Kienast J, Schmitz G. Flow cytometric analysis of thiazole orange uptake by platelets: a diagnostic aid in the evaluation of thrombocytopenic disorders. *Blood* 1990; 75: 116–121.

4 Takubo T, Yamane T, Hino M et al. Usefulness of determining reticulated and large platelets in idiopathic thrombocytopenic purpura. *Acta Haematol* 1998; 99: 109–110.

5 Saxon BR, Blanchette VS, Butchart S et al. Reticulated platelet counts in the diagnosis of acute immune thrombocytopenic purpura. *J Pediatr Hematol Oncol* 1998; 20: 44–48.

6 Kurata Y, Hayashi S, Kiyoi T et al. Diagnostic value of tests for reticulated platelets, plasma glycocalicin, and thrombopoietin levels for discriminating between hyperdestructive and hypoplastic thrombocytopenia. *Am J Clin Pathol* 2001; 115: 656–664.

7 Kunishima S, Tahara T, Kato T et al. Serum thrombopoietin and plasma glycocalicin concentrations as useful diagnostic markers in thrombocytopenic disorders. *Eur J Haematol* 1996; 57: 68–71.

8 Kosugi S, Kurata Y, Tomiyama Y et al. Circulating thrombopoietin level in chronic immune thrombocytopenic purpura. *Br J Haematol* 1996; 93: 704–706.

9 Porcelijn L, Folman CC, Bossers B et al. The diagnostic value of thrombopoietin level measurements in thrombocytopenia. *Thromb Haemost* 1998; 79: 1101–1105.

10 Steffan A, Pradella P, Cordiano I et al. Glycocalicin in the diagnosis and management of immune thrombocytopenia. *Eur J Haematol* 1998; 61: 77–83.

11 Kelton JG. The serological investigation of patients with autoimmune thrombocytopenia. *Thromb Haemost* 1995; 74: 228–233.

12 Warner M, Kelton JG. Laboratory investigation of immune thrombocytopenia. *J Clin Pathol* 1997; 50: 5–12.

13 Chong BH, Keng TB. Advances in the diagnosis of idiopathic thrombocytopenic purpura. *Semin Hematol* 2000; 3: 249–260.

14 Mueller-Eckhardt C, Kayser W, Mersch-Baumert K et al. The clinical significance of platelet-associated IgG: a study on 298 patients with various disorders. *Br J Haematol* 1980; 46: 123–131.

15 Kelton JG, Powers P, Carter C. A prospective study of the usefulness of the measurement of platelet-associated IgG for the diagnosis of idiopathic thrombocytopenic purpura. *Blood* 1982; 60: 1050–1053.

16 Brighton T, Evans S, Castaldi PA et al. Prospective evaluation of the clinical usefulness of an antigen-specific assay (MAIPA) in idiopathic thrombocytopenic purpura and other immune thrombocytopenias. *Blood* 1996; 88: 194–201.

Cambridge Books Online © Cambridge University Press, 2009

17 Warner MN, Moore JC, Warkentin TE et al. A prospective study of protein-specific assay used to investigate idiopathic thrombocytopenic purpura. *Br J Haematol* 1999; 104: 442–447.
18 Marcaccio MJ. Laparoscopic splenectomy in chronic idiopathic thrombocytopenic purpura. *Semin Hematol* 2000; 37: 267–274.
19 Stasi R, Stipa E, Masi M et al. Long-term observation of 208 adults with chronic idiopathic thrombocytopenic purpura. *Am J Med* 1995; 98: 436–442.
20 DiFino SM, Lachant NA, Kirshner JJ, Gottlieb AJ. Adult idiopathic thrombocytopenic purpura: clinical findings and response to therapy. *Am J Med* 1980; 69: 430–442.
21 Ambriz R, Munoz R, Quintanar E et al. Accessory spleen compromising response to splenectomy for idiopathic thrombocytopenic purpura. *Radiology* 1985; 155: 793–796.
22 Gibson J, Rickard KA, Bautovich G et al. Management of splenectomy failures in chronic immune thrombocytopenic purpura: role of accessory splenectomy. *Aust N Z J Med* 1986; 16: 695–698.
23 Ahn YS, Harrington WJ, Byrnes JJ et al. The treatment of idiopathic thrombocytopenia with vinblastine-loaded platelets. *N Engl J Med* 1978; 298: 1101–1107.
24 Nenci GG, Agnelli G, De Cunto M, Gresele P. Infusion of Vincristine-loaded platelets in acute ITP refractory to steroids: an alternative to splenectomy. *Acta Haematol* 1981; 66: 117–121.
25 Ahn YS, Harrington WJ, Mylvaganam R et al. Slow infusion of *Vinca* alkaloids in the treatment of idiopathic thrombocytopenic purpura. *Ann Intern Med* 1984; 100: 192–196.
26 Emilia G, Longo G, Luppi M et al. *Helicobacter pylori* eradication can induce platelet recovery in idiopathic thrombocytopenic purpura. *Blood* 2001; 97: 812–814.
27 Saleh MN, Gutheil J, Moore M et al. A pilot study of the anti-CD20 monoclonal antibody rituximab in patients with refractory immune thrombocytopenia. *Semin Oncol* 2000; 27(6 Suppl 12): 99–103.
28 Grossi A, Santini V, Longo G et al. Treatment with anti-CD20 antibodies of patients with autoimmune thrombocytopenia with or without hemolytic anemia; worsening in hemoglobin level (abstract). *Blood* 2000; 96: 253a.
29 Bussel JB, Mukherjee R, Stone AJ. A pilot study of rhuIL-11 treatment of refractory ITP. *Am J Hematol* 2001; 66: 172–177.
30 Terjanian T, Sher H, Vemuri R et al. A proof-of-concept phase 2 study of monoclonal antibody MDX-33 in adult subjects with chronic stable ITP (abstract). *Blood* 2000; 96: 251a.
31 Winkelmann JC, Hawley D, Flaspohler. Taxol is highly active in refractory ITP (abstract). *Blood* 2000; 96: 250a.
32 Snyder HW Jr, Cochran SK, Balint JP Jr et al. Experience with protein A-immunoadsorption in treatment-resistant adult immune thrombocytopenic purpura. *Blood* 1992; 79: 2237–2245.
33 Fabrizio F, Luzzatto G, Ramon R et al. Treatment of refractory ITP with extracorporeal immunoadsorption over a protein-A sepharose column: a report of two cases. *Haematologica* 2000; 85: 889–890.
34 Burrows RF, Kelton JG. Fetal thrombocytopenia and its relation to maternal thrombocytopenia. *N Engl J Med* 1993; 329: 1463–1466.
35 Crowther MA, Burrows RF, Ginsberg J, Kelton JG. Thrombocytopenia in pregnancy: diagnosis, pathogenesis and management. *Blood Rev* 1996; 10: 8–16.
36 Gill KK and Kelton JG. Management of idiopathic thrombocytopenic purpura in pregnancy. *Semin Hematol* 2000; 3: 275–289.
37 Cines DB, Dusak B, Tomaski A et al. Immune thrombocytopenic purpura and pregnancy. *N Engl J Med* 1982; 306: 826–831.
38 Hart D, Dunetz C, Nardi M et al. An epidemic of maternal thrombocytopenia associated with elevated antiplatelet antibody. Platelet count and antiplatelet antibody in 116 consecutive pregnancies: Relationship to neonatal platelet count. *Am J Obstet Gynecol* 1986; 154: 878–883.
39 Burrows RF, Kelton JG. Thrombocytopenia at delivery: A prospective survey of 6715 deliveries. *Am J Obstet Gynecol* 1990; 162: 731–734.
40 Burrows RF, Kelton JG. Low fetal risks in pregnancies associated with idiopathic thrombocytopenic purpura. *Am J Obstet Gynecol* 1990; 163: 1147–1150.
41 Moutet A, Fromont P, Farcet JP et al. Pregnancy in women with immune thrombocytopenic purpura. *Arch Intern Med* 1990;150: 2141–2145.
42 Samuels P, Bussel JB, Braitman LE et al. Estimation of risk of thrombocytopenia in the offspring of pregnant women with presumed immune thrombocytopenic purpura. *N Engl J Med* 1990; 323: 229–235.
43 Cook RL, Miller RC, Katz VL et al. Immune thrombocytopenic purpura in pregnancy: a reappraisal of management. *Obstet Gynecol* 1991; 78: 578–583.
44 Sainio S, Joutsi L, Jarvenpaa AL et al. Idiopathic thrombocytopenic purpura in pregnancy. *Acta Obstet Gynecol Scand* 1998; 77: 272–277.
45 Valet AS, Caulier MT, Devos P et al. Relationships between severe neonatal thrombocytopenia and maternal characteristics in pregnancies associated with autoimmune thrombocytopenia. *Br J Haematol* 1998; 103: 397–401.
46 Yamada H, Kato EH, Kobashi G et al. Passive immune thrombocytopenia in neonates of mothers with idiopathic thrombocytopenic purpura: Incidence and risk factors. *Semin Thromb Hemost* 1999; 25: 491–496.
47 Boehlen F, Hohlfeld P, Extermann P et al. Maternal antiplatelet antibodies in predicting risk of neonatal thrombocytopenia. *Obstet Gynecol* 1999; 93: 169–173.
48 Bussel JB, Christiaens G. Birth platelet counts in sequential newborns of mothers with ITP: Do the platelet counts change with subsequent babies? *Blood* 1993; 82(suppl. 1): 202a.
49 Christiaens GCML, Nieuwenhuis HK, Bussel JB. Comparison of platelet counts in first and second newborns of mothers with immune thrombocytopenic purpura. *Obstet Gynecol* 1997; 90: 546–552.
50 Payne SD, Resnik R, Moore TR et al. Maternal characteristics and risk of severe neonatal thrombocytopenia and intracranial hemorrhage in pregnancies complicated by autoimmune thrombocytopenia. *Am J Obstet Gynecol* 1997; 177: 149–155.

Cambridge Books Online © Cambridge University Press, 2009

51 Sloand EM, Klein HG, Banks SM et al. Epidemiology of thrombocytopenia in HIV infection. *Eur J Haematol* 1992; 48: 168–172.
52 Walsh C, Krigel R, Lennette E, Karpatkin S. Thrombocytopenia in homosexual patients. Prognosis, response to therapy, and prevalence of antibody to the retrovirus associated with the acquired immunodeficiency syndrome. *Ann Intern Med* 1985; 103: 542–545.
53 Marroni M, Gresele P. Detrimental effects of high-dose dexamethasone in severe, refractory, HIV-related thrombocytopenia. *Ann Pharmacother* 2000; 34: 1139–1141.
54 Gillis S, Eldor A. Immune thrombocytopenic purpura in adults: clinical aspects. *Baillieres Clin Haematol* 1998; 11: 361–372.
55 Coyle TE. Hematologic complications of human immunodeficiency virus infection and the acquired immunodeficiency syndrome. *Med Clin North Am* 1997; 81: 449–470.
56 Marroni M, Gresele P, Vezza R et al. Thrombocytopenia in HIV infected patients. Prevalence and clinical spectrum. *Recenti Prog Med* 1995; 86: 103–106.
57 Marroni M, Gresele P, Landonio G et al. Interferon-alpha is effective in the treatment of HIV-1-related, severe, zidovudine-resistant thrombocytopenia. A prospective, placebo-controlled, double-blind trial. *Ann Intern Med* 1994; 121: 423–429.
58 Budman DR, Steinburg AD. Hematologic aspects of systemic lupus erythematosus. Current concepts. *Ann Intern Med* 1977; 86: 220–229.
59 Hall S, McCormick JL Jr, Greipp PR et al. Splenectomy does not cure the thrombocytopenia of systemic lupus erythematosus. *Ann Intern Med* 1985; 102: 325–328.
60 McNeil HP, Simpson RJ, Chesterman CN, Krilis SA. Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: β2-glycoprotein I (apolipoprotein H). *Proc Natl Acad Sci USA* 1990; 87: 4120–4124.
61 Cuadrado MJ, Mujic F, Munoz E et al. Thrombocytopenia in the antiphospholipid antibody syndrome. *Ann Rheum Dis* 1987; 56: 194–196.
62 Galli M, Finazzi G, Barbui T. Thrombocytopenia in the antiphospholipid syndrome: pathophysiology, clinical relevance and treatment. *Ann Med Interne* 1996; 147(Suppl 1): 24–27.
63 Munoz-Rodriguez FJ, Font J, Cervera R et al. Clinical study and follow-up of 100 patients with the antiphospholipid antibody syndrome. *Semin Arthritis Rheum* 1999; 29: 182–190.
64 Kaden BR, Rosse WF, Hauch TW. Immune thrombocytopenia in lymphoproliferative diseases. *Blood* 1979; 53: 545–551.
65 Rudders RA, Aisenberg AC, Schiller AL. Hodgkin's disease presenting as 'idiopathic' thrombocytopenic purpura. *Cancer* 1972; 30: 220–230.
66 West KA, Anderson DR, McAlister VC et al. Alloimmune thrombocytopenia after organ transplantation. *N Engl J Med* 1999; 341: 1504–1507.
67 Bussel JB, Zabusky MR, Berkowitz RL, McFarland JG. Fetal alloimmune thrombocytopenia. *N Engl J Med* 1997; 337: 22–26.
68 Porcelijn L, von dem Borne AEGKr. Immune-mediated thrombocytopenias: basic and immunological aspects. *Baillieres Clin Haematol* 1998; 11: 331–341.
69 L'Abbe D, Tremblay L, Filion M et al. Alloimmunization to platelet antigen HPA-1a (PLA1) is strongly associated with both HLA-DRB3*0101 and HLA-DQB1*0201. *Hum Immunol* 1992; 34: 107–114.
70 Brockmeir FK, Nordhagen R, Finne PH. Neonatal alloimmune thrombocytopenia. Case reports, diagnostic and therapeutic considerations. *Acta Paediatr Scand* 1983; 72: 583–587.
71 Warkentin TE, Kelton JG. Heparin and Platelets. *Hematol Oncol Clin North Am* 1990; 4: 243–264.
72 King DJ, Kelton JG. Heparin-associated thrombocytopenia. *Ann Intern Med* 1984; 100: 535–540.
73 Amiral J, Bridey F, Dreyfus M et al. Platelet factor 4 complexed to heparin is the target for antibodies generated in heparin-induced thrombocytopenia. *Thromb Haemost* 1992; 68: 95–96.
74 Kelton JG, Smith JW, Warkentin TW et al. Immunoglobulin G from patients with heparin-induced thrombocytopenia binds to a complex of heparin and platelet factor 4. *Blood* 1994; 83: 3232–3239.
75 Kelton JG, Sheridan D, Santos A et al. Heparin-induced thrombocytopenia: laboratory studies. *Blood* 1988; 72: 925–930.
76 Warkentin TE, Hayward CPM, Boshkov LK et al. Sera from patients with heparin-induced thrombocytopenia generate platelet-derived microparticles with procoagulant activity: an explanation for the thrombotic complications of heparin-induced thrombocytopenia. *Blood* 1994; 84: 3691.
77 Warkentin TE, Sheppard JA, Horsewood P et al. Impact of the patient population on the risk for heparin-induced thrombocytopenia. *Blood* 2000; 96: 1703–1708.
78 Sheridan D, Carter C, Kelton JG. A diagnostic test for heparin-induced thrombocytopenia. *Blood* 1986; 67: 27–30.
79 Warkentin TE, Kelton JG. The platelet lifecycle: qualitative disorders. In Handin RI, Lux SE, Stossel TP, eds. *Blood: Principles and Practice of Hematology*. Philadelphia: J.B. Lippincott Company; 1995: 973–1048.
80 Adachi JD, Bensen WG, Kassam Y et al. Gold induced thrombocytopenia: 12 cases and a review of the literature. *Semin Arthritis Rheum* 1987;16: 287–293.
81 Herbst KD, Stone WH, Flannery EP. Letter: Chronic Thrombocytopenia following gold therapy. *Arch Intern Med* 1975; 135: 1622.
82 England JM, Smith DS. Gold-induced thrombocytopenia and response to dimercaprol. *Br Med J* 1972; 2: 748–749.
83 Stafford BT, Crosby WH. Late onset of gold-induced thrombocytopenia. With a practical note on the injections of dimercaprol. *JAMA* 1978; 239: 50–51.
84 Godfrey NF, Peter A, Simon TM, Lorber A. IV N-acetylcysteine treatment of hematologic reactions to chrysotherapy. *J Rheumatol* 1982; 9: 519–526.
85 Berndt MC, Chong BH, Bull HA et al. Molecular characterization of quinine/quinidine drug-dependent antibody platelet interaction using monoclonal antibodies. *Blood* 1985; 66: 1292–1301.

Cambridge Books Online © Cambridge University Press, 2009

37

Thrombocytopenia in childhood

C. Kaplan, M. Dreyfus, V. Proulle, G.Tchernia

Unité d'Immunologie Plaquettaire, INTS, Paris, France

Thrombocytopenia in children as in adults has a pleiomorphic expression, which may vary from a life-threatening hemorrhagic accident, to a biological finding in an apparently healthy child. In many patients clinical expression is absent or mild and thrombocytopenia will be fortuitously detected by blood cell counts during the course of a disease or prior to surgery. Neonatal thrombocytopenias also have special features requiring special diagnostic approach and management.

Platelet counting by blood cell automatic counters usually is reliable. However, in some situations, platelet counting can be erroneous; for example, circulating particles whose volume is within the platelet volume range can spuriously increase the platelet count and mask a thrombocytopenia; this can occur with extreme microcytosis or red cell fragmentation. On the contrary, in vitro platelet consumption, due to hemostasis activation during sampling or inefficient anticoagulation of the sample or to EDTA-induced platelet aggregation[1], can lead to spurious thrombocytopenia. In any case, an initial diagnosis of thrombocytopenia must be verified by phase contrast microscopy after microsampling, and by examination of the platelets' size, morphology and density on blood smears. There is no close correlation between clinical presentation and platelet count. Most patients will have minimal bleeding symptoms even with platelet counts below 50×10^9/l. Others can exhibit bruising and bleeding in spite of moderate thrombocytopenia due to associated platelet functional abnormalities. The search for a precise etiology, which can be either constitutional or acquired, needs a rigorous approach, as therapeutic decisions have to rely on certainties (Table 37.1).

In this chapter drug-induced thrombocytopenia and thrombocytopenia associated with malignancies will not be considered.

Diagnostic approaches to thrombocytopenia in infancy and childhood

Thrombocytopenia is defined by a platelet count less than 150×10^9/l. Due to the difficulties already described, blood smear examination, micro-sampling, phase microscopy, coagulation assessment and repeated platelet counts are mandatory to confirm the diagnosis, whatever the patient age. As in adults, thrombocytopenia results either from increased platelet destruction or decreased platelet production. This distinction can be difficult as in many disorders multiple factors can be involved in the etiology of thrombocytopenia.

Furthermore, the distinction between these two mechanisms may be difficult to achieve especially in neonates.

(i) Blood sampling is often difficult, and the small volume available allows limited biological studies.
(ii) Bone marrow aspiration and biopsy are technically difficult and megakaryocytes at birth can be located elsewhere than in the bone marrow. Being more and more aware of the importance of pain in very young infants, we are prone to avoid such investigations unless they appear absolutely necessary.
(iii) Radioactive studies of platelet kinetics are not usually performed in infants.

Determination of thrombopoietin (Tpo) levels can be of help for differential diagnosis of thrombocytopenia. Tpo, also known as Mpl ligand, is considered to be the major regulator of thrombopoiesis, promoting proliferation, growth and development of megakaryocytes from CD34+ cells, polyploïdization and proplatelet formation. Studies have been performed in the fetus and neonate to assess Tpo levels in normal and thrombocytopenic state. It has been shown that Tpo is present in higher concentrations in cord blood than in adults' serum samples and that the level does not correlate with either gestational age or platelet

Table 37.1. Etiology of thrombocytopenia in childhood

Acquired thrombocytopenias	Immunologic processes	Autoimmunity	AITP
			Neonatal thrombocytopenia due to maternal autoimmunity
		Alloimmunization	Neonatal alloimmune thrombocytopenia
	Non-immune processes	Infections	
		Increased platelet consumption	Disseminated intravascular coagulation
			Hemolytic uremic syndrome
			Thrombotic thrombocytopenic purpura
			Kasabach–Meritt syndrome
			Miscellaneous causes
Inherited thrombocytopenias		Small platelet size	Wiskott–Aldrich Syndrome
			X-linked thrombocytopenia
		Inherited giant platelet disorders	May–Hegglin disease, Alport syndrome, Fechtner syndrome
			Bernard–Soulier syndrome
			Grey platelet syndrome
			von Willebrand 2B syndrome
		Normal platelet volume	Thrombocytopenia with absent radii (TAR) syndrome
			Congenital amegakaryocytic thrombocytopenia
			Paris–Trousseau thrombocytopenia

count at birth[2]. A majority of neonates with hypogenerative thrombocytopenia have low levels of Tpo when compared to those reported in adults[3,4]. Appropriate references must be considered if Tpo assays are used for distinguishing thrombocytopenia associated with decreased platelet production from thrombocytopenia resulting from increased platelet destruction.

In these neonatal cases, it is therefore of prime importance to collect any available information, which may assist in diagnosis: maternal history of thrombocytopenia, recent bacterial infection or disseminated intravascular coagulation (DIC) must be searched for. Maternal platelet counts and neonatal coagulation tests should be available. A previous history of neonatal thrombocytopenia in the siblings or relatives is important and in some cases immunological tests for maternofetal alloimmunization should be carried out.

In childhood, acute autoimmune thrombocytopenic purpura (AITP) is the most frequent cause of thrombocytopenia and the diagnosis is usually made on clinical evidence after exclusion of other obvious causes:

(i) the sudden occurrence of a severe thrombocytopenia, associated with an extensive purpura and/or mucosal hemorrhage in an otherwise healthy child;
(ii) a recent viral infection/ vaccination in the child or a sibling.

The presence of an isolated, severe thrombocytopenia usually confirms the diagnosis. A bone marrow study is mandatory only in cases of atypical clinical or biological features, or for some authors before a steroid therapy is instituted in order to rule out a leukemia or a myelodysplasic syndrome.

The detection of specific autoantibodies using antigen-capture assays (Immunobead assay[5], MAIPA test[6,7]) is not mandatory, but may be of help in case of equivocal diagnosis. The indirect MAIPA is less likely to be positive in AITP than the direct MAIPA[8]. However, these assays are not easily available for routine diagnosis.

As in neonates, Tpo assays can be considered in the differential diagnosis to determine the mechanism of the thrombocytopenia[9]. The results must be compared to the normal range defined according to age.

In the case of chronic thrombocytopenia, other investigations, such as isotopic studies, are required to further confirm the diagnosis before therapeutic decisions such as splenectomy are made.

Platelet aggregation studies, vWF cofactor activity assays, platelet counts and vWF assessment in the parents and siblings, and platelet glycoprotein studies will be performed in cases of suspected inherited thrombocytopenia.

Cambridge Books Online © Cambridge University Press, 2009

Acquired thrombocytopenia

Immunologic processes

Autoimmunity

Neonatal thrombocytopenia due to maternal autoimmune thrombocytopenic purpura

Autoimmune thrombocytopenic purpura (AITP) in pregnant women can induce moderate or severe thrombocytopenia in the fetus or in the newborn whatever the mother's disease status. It may occur notwithstanding splenectomy, in babies of thrombocytopenic mothers or of mothers with normal platelet counts due to compensated thrombocytolysis.

It is unpredictable and occurs in 30–40% of cases. For most authors none of the mother's clinical or biological parameters can predict the risk of thrombocytopenia in the newborn[10]. However, more recently studies have shown that severe neonatal thrombocytopenias are more often found in offspring of women with previous splenectomy[11]. Furthermore, mothers with AITP and HLA DRB3* phenotype seem to be protected against giving birth to a thrombocytopenic newborn; in contrast HLA DR02, DR5* could provide a higher risk. This last report, if confirmed by a multicentric study, could constitute a predictive factor useful in the management of pregnancy and of the newborn[12]. It has been shown that, despite the absence of prenatal bleeding in most cases, thrombocytopenia could be found as early as 20 weeks of gestational age and increases as gestation proceeds. Fetal thrombocytopenia cannot be prevented: intravenous immunoglobulins (IVIgG) or steroids, even if effective in raising the mother's platelet counts, do not appear to be able to decrease fetal platelet destruction[13].

The mode of delivery of mothers with AITP has evolved during the past years. Cesarean sections have been advocated to avoid potential intracranial hemorrhage in severely thrombocytopenic fetuses, whereas vaginal delivery has been suggested to bear a higher risk. In order to determine the mode of delivery and to decrease the rate of unnecessary cesarean sections, percutaneous umbilical blood sampling has been advocated. More recently the rationale for the assessment of fetal platelet counts has been subject to controversy.

(i) Up to now there is no prospective study showing that cesarean section is more effective in preventing intracranial hemorrhage than vaginal delivery.
(ii) In pregnant women with AITP, severe fetal thrombocytopenia is observed in only 10 to 15% of the cases.
(iii) Not all fetuses with severe antenatal thrombocytopenia will suffer intracranial bleeding.
(iv) Percutaneous umbilical blood sampling (PUBS) has a 0.5–1.5% complication rate.

Therefore PUBS is not recommended in this situation unless there has been severe bleeding in the offspring. In these cases, PUBS, if decided, must be performed in a referral centre and at the end of pregnancy. In our practice, in case of obstetrical complications and suspicion of severe fetal thrombocytopenia, we propose cesarean delivery as an alternative.

After birth, close monitoring of the newborn platelet count is recommended. Eventuality of an associated impairment of platelet function due to the interaction of autoantibodies with their epitope must not be ignored, and neonatal bleeding associated with moderate thrombocytopenia has been reported in such cases. Neonatal thrombocytopenia usually worsens during the first days of life with a nadir on day 3 to 5 and lasts from 10 to 60 days. Postnatal management includes IVIgG which has been shown to be effective most of the time, and low dose steroid therapy, which may be prescribed as an hemostatic agent. Exchange transfusion has only a moderate and transient effect but can be proposed in case of severe bleeding (Table 37.2).

Table 37.2. Neonatal thrombocytopenia due to maternal autoimmune thrombocytopenic purpura (AITP)

The fetal/neonatal thrombocytopenia is unpredictable:
30–40% of infants born to mothers with AITP will be thrombocytopenic
10–15% of infants will be severely thrombocytopenic but only 0–3% of infants with intracranial hemorrhage
No antenatal therapy to reverse fetal thrombocytopenia
Delivery:
vaginal delivery in most cases
cesarean section in case of obstetrical complications and suspicion of severe fetal thrombocytopenia
After birth:
close monitoring of the newborn platelet count
nadir of the neonatal thrombocytopenia on day 3–5
neonatal thrombocytopenia observed from 10 to 60 days
therapy: IVIgG, exchange-transfusion in case of severe bleeding

Acute AITP in childhood

Acute AITP is a frequent disease occurring in most cases between 2 and 8 years of age. It is generally a benign, self-limited condition with, in 80–90% of cases, a spontaneous

Cambridge Books Online © Cambridge University Press, 2009

recovery within a matter of days or weeks. The incidence has been reported to be 5.3 per 1 000 000 children under 15 years[14]. It often follows a seasonal viral illness, most often upper respiratory tract infection occurring within the 4 weeks before the child presents with thrombocytopenia. Vaccination, infectious mononucleosis, varicella, or various non-diagnosed erythemas can also be incriminated[15]. The male : female ratio is 1. Bruising and mucosal hemorrhage are often the first symptoms with an abrupt onset. The characteristic features are isolated thrombocytopenia and normal to increased numbers of megakaryocytes in the bone marrow[16]. The severity of thrombocytopenia reflects the balance between antibody-mediated platelet destruction and increased platelet production from megakaryocytes. The risk of severe bleeding is correlated with the clinical expression: nasal bleeds, menorrhagias in girls, hemorrhagic bullae inside the mouth on the internal face of the cheek, can alert to the potential severity of the disease.

The association with any coagulation abnormality can enhance the risk for bleeding and call for adapted management. Storing frozen serum samples can be of a great help for further immunological or viral investigations, which might not appear to be of interest at presentation. Bone marrow aspirate is recommended in all children with acute AITP if clinical presentation or laboratory features are atypical and in those children treated with IVIgG or short-term oral steroids who either fail to respond or require retreatment[17]. Approximately 10–20% of children with acute AITP will develop the chronic variety (Table 37.3).

Table 37.3. Autoimmune thrombocytopenic purpura (AITP) in childhood

Acute AITP
frequent disease between 2 and 8 years of age
generally benign with in 80–90% of cases a spontaneous recovery
viral infections have been incriminated
abrupt onset of bruising and mucosal hemorrhage
isolated thrombocytopenia with normal to increased numbers of megakaryocytes
bone marrow aspirate is recommended in all children if clinical presentation or laboratory features are atypical or in case of failure therapy with IVIgG or short-term oral steroids
therapy is controversial: steroids, IVIgG
Chronic AITP
disease persisting for more than 6 months
intracranial hemorrhage in less than 0.1% of the cases and in 0.5% of the cases with a platelet count persistently $<20\times10^9$/l 12 months after the diagnosis
therapy: IVIgG steroids; splenectomy has to be restricted to particular cases with refractoriness to all medical treatments, persistent bleeding tendency, risk for cerebral hemorrhage or unacceptable side effects of medical therapy
Emergency therapy
for patients with significant mucosal bleeding or extremely low platelet counts
hospitalization and therapy with a combination of IV steroids, IVIgG and platelet transfusion

Chronic AITP

Chronic AITP implies disease persisting for more than 6 months, which is an arbitrary threshold. The diagnosis of chronic AITP is mainly a process of exclusion, which is based on considering non-immune causes of long-term isolated thrombocytopenia such as various congenital thrombocytopenias and type 2B von Willebrand disease and also excluding immune-mediated thrombocytopenia associated with multisystem autoimmune disease or more generalized immune dysfunction[18]. A major difference will exist between children with repeated clinical manifestations and those who disclose only laboratory evidence of persistent antiplatelet autoimmunity, with no hemorrhagic symptoms[18]. Some features have been claimed to be predictive of the development of a chronic form of AITP, such as insidious presentation, female sex, age over 10 years, symptoms lasting for more than 2 weeks after diagnosis[19]. The incidence of life-threatening hemorrhage is lower than previously reported. Intracranial hemorrhage occurs in less than 0.1% of the cases and in 0.5% of the cases with a platelet count persistently $<20\times10^9$/l 12 months after the diagnosis[20]. There may be associated predisposing factors such as trauma or vascular malformations.

Most importantly, thrombocytopenia will resolve or switch from a severe to a mild form in the majority of children with chronic AITP, with an estimate of 30% at 2 years and 61% after 15 years[21] (Table 37.3).

Therapy of AITP in childhood

The therapeutic decision should mostly rely on clinical severity, but most surveys have shown a generalized tendency to treat platelet counts rather than bleeding symptoms[14,22] (Table 37.3).

Acute AITP

The aim of the treatment is only to shorten the risk period by increasing the platelet count before a spontaneous increase takes place. Initial treatment does not change the total duration of the illness and does not reduce the number of patients whose disease becomes chronic. Most

Cambridge Books Online © Cambridge University Press, 2009

authors consider that in AITP platelet counts above 30×10^9/l without overt cutaneous or mucosal hemorrhage do not require treatment in the absence of trauma or need for surgery. The decision of initial therapy is based individually on a combination of platelet count and clinical factors. Controversy exists about most published guidelines[18,23–25]. Owing to the rarity of intracranial bleeding, it has been suggested by some authors that initial treatment should not be prescribed in most cases, provided the patients are under close control until the platelet count begins to rise. A subgroup of children should, in any case, be treated: those with platelet count $<20 \times 10^9$/l, if additional risk factors such as head trauma or recent use of aspirin are identified. If a treatment is decided, the minimum therapy necessary to increase the platelet count to a safe level should be prescribed[26]. For many years, most pediatricians have used prednisone at a dose of 1–2 mg/kg as initial treatment for 10–28 days[27]. In most cases this leads to a stable increase in platelet counts within a few days and to a more rapid disappearance of petechiae and bleeding symptoms. When the dose is tapered, the platelet count can drop and clinical expression may reappear in some patients: in that case both evolution towards chronic AITP and subsequent spontaneous resolution can be observed. In any case, steroids should never be given for a prolonged period and, should the evolution be prolonged, other therapeutic approaches must be proposed. Short-term oral prednisone (4 mg/kg/day $\times$ 4 days) is probably a good alternative[28].

IVIgG has been shown to increase transiently the platelet count in patients with AITP[29,30]. The most frequently recommended dosage has been 0.8 g/kg/day[26]. The increase can occur in the 12–72 hours following the infusion. In most cases of acute AITP, a spontaneously favourable outcome is reached during the time of the therapeutic response. The major limitations are the price of the product, the time necessary for its administration in outpatients, the problems of viral safety, and some side effects such as meningitis or renal complications[26,31,32]. Platelet transfusions are not a treatment of immune AITP. However they may be considered, in conjunction with other treatments, for patients in whom a rapid rise in platelet count is deemed essential, that is before surgery, after significant trauma, or because of life-threatening mucosal or internal hemorrhage[25,27]. Intravenous rhesus antibodies have been shown to be less effective than steroid or IVIgG in acute AITP and should not be used as a first-line therapy[26].

Chronic AITP

Prior to any treatment, the diagnosis has to be firmly established in order to avoid potentially harmful therapy for other diseases, mostly constitutional thrombocytopenias[33], SLE- or HIV-related thrombocytopenias. In most cases a sharp rise is obtained with high dose IVIgG but the response duration will not exceed 1–6 weeks before relapse occurs. However, IVIgG is of major interest in two settings: (i) when a transient increase in platelet count is necessary, e.g. for surgery; (ii) in some patients regular IVIgG infusions can maintain a 'safe' platelet count and a normal life for several months, with an injection on an outpatient basis every 3–6 weeks. Moreover, in some of these patients the interval can gradually be increased. This strategy can provide the necessary time to learn the degree of tolerance and the natural history of the disease before discussing splenectomy or waiting for a favourable evolution.

Splenectomy results in a much higher cure rate than any medical regimen. The overall response rate averages 70%, with 14% relapse. An additional 12% of these patients achieve a stable partial response. Therefore, about 72% of patients have a beneficial response. According to various reports, several features could help to predict the result, such as previous steroid responsiveness, short survival and high turnover of labelled platelets[34] or high postoperative rise in platelet count. However, there is no foolproof way to predict which individual patient will have a stable response to splenectomy. Some surgical failures or relapses can be related to a persistent accessory spleen.

Operative morbidity of splenectomy is uncommon. If steroid therapy or IVIgG has previously been shown to be transiently efficient, they should be prescribed before surgery in order to allow a safe procedure. Otherwise, platelet concentrates should be ready for use and infused immediately if surgical hemostasis is difficult to achieve. However, the hazard of severe infections postsplenectomy has severely restrained the indications for splenectomy in all conditions including AITP. It should be emphasized that, in a large series of AITP in children, the mortality risk of overwhelming postsplenectomy infection can be as important as that of CNS hemorrhage[35]. In any case, all patients, whatever their age, should receive appropriate vaccination before surgery and regular oral penicillin prophylaxis should be prescribed. This particular risk explains why splenectomy, which everyone agrees to be the most frequently effective treatment, has been restricted to particular cases with refractoriness to all medical treatments, persistent bleeding tendency, risk for cerebral hemorrhage, or unacceptable side effects of medical treatments. Anti-D immunoglobulin in non-splenectomized Rhesus-positive patients with chronic ITP can result in a transient rise in the platelet count[17,36].

Cambridge Books Online © Cambridge University Press, 2009

Refractory chronic AITP

There is at present no definitive treatment for refractory chronic AITP. The plethora of suggested solutions simply reflects the fact that refractory idiopathic thrombocytopenic purpura often remains refractory[17,37].

Emergency therapy

Whatever the clinical course of AITP, acute or chronic, patients with significant mucosal bleeding or extremely low platelet counts ($<5\times10^9$–10×10^9/l) must be considered as at risk for severe bleeding, and immediately hospitalized and treated by a combination of IV steroids, IVIgG and platelet transfusion.

Alloimmunization

Immunization against platelet alloantigens or HPA (human platelet antigen) can occur either during pregnancy or after transfusion, leading, respectively, to neonatal alloimmune thrombocytopenia or post-transfusion purpura (PTP). However, PTP, which appears to be an anamnestic immune response, has not been described in children.

Neonatal alloimmune thrombocytopenia (NAIT) results from maternal allo-immunization against fetal platelet antigens inherited from the father, which she herself lacks. The incidence of feto-maternal alloimmunization (FMAIT) is estimated to be 1 per 800 to 1000 live births[38,39]. NAIT is considered to be the counterpart of Rh hemolytic disease of the newborn (HDN). In contrast to HDN, neonatal alloimmune thrombocytopenia may affect the first child. There may be severe bleeding in the central nervous system (CNS) and death or severe neurological sequelae.

Progress in platelet immunology and fetal medicine has led to the development of antenatal therapy and better management of this high-risk group, which are still evolving (Table 37.4).

The usual presentation is a neonate born to a healthy mother, who exhibits widespread purpura at or a few hours after birth. Otherwise, this infant is healthy, with no clinical signs of infection or malformation. Visceral hemorrhages are less common. The most serious complication is ICH leading to death in up to 10% of the reported cases, or to neurological sequelae in 20% of the cases[40,41]. Such hemorrhages may occur as early as 16 weeks of gestation and induce irreversible cerebral lesions. They may be present at birth or can occur as long as the newborn is thrombocytopenic. The risk of life-threatening hemorrhage necessitates prompt diagnosis and effective therapy.

The diagnosis of NAIT is made initially on clinical grounds and depends upon the exclusion of other causes of neonatal thrombocytopenia.

Table 37.4. Alloimmunization

Neonatal alloimmune thrombocytopenia results from maternal alloimmunization against a fetal platelet antigen inherited from the father, which she herself lacks

Incidence 1 per 800 to 1000 live births

Clinical presentation

neonate born to a healthy mother who exhibits widespread purpura at or a few hours after birth
no clinical sign of infection or malformation
visceral hemorrhages are less common
the most serious complication is intracranial hemorrhage leading to death in up to 10% of the reported cases or to 20% of neurological sequelae

Diagnosis

clinical grounds and exclusion of other causes of neonatal thrombocytopenia
isolated thrombocytopenia
platelet immunological testing: parental platelet alloantigen incompatibility with a corresponding maternal antibody

Therapy

transfusion of platelets compatible with the maternal alloantibody, the mother is the most reliable donor

Management of subsequent pregnancies

the optimal therapy is a matter of debate
recommendations based on our practice

* high-risk women must be followed in a referral centre with fetal blood sampling performed in case of incompatible fetus
* maternal therapy with IVIgG± corticosteroids in case of fetal thrombocytopenia
* cesarean section in case of therapy failure

The neonatal platelet count is low and anemia is only seen when secondary to bleeding. Platelet immunological investigations must be performed in a well-versed laboratory because of possible difficulties. The testing involves the detection of maternal circulating antibody and identification of the offending platelet antigen with the determination of the parents' platelet genotype. Detection of antibodies is usually done with antigen capture ELISA[6]. Molecular techniques are used for genotyping[42].

The diagnosis is straightforward when a parental antigen incompatibility with a corresponding maternal antibody are present. It could be equivocal in the absence of such an antibody or when a new, rare or private antigen is implicated. Any difficulties in confirming the diagnosis should not delay therapy when there are sufficient grounds for a provisional diagnosis.

Until there is a prospective screening programme for

Cambridge Books Online © Cambridge University Press, 2009

NAIT, most of the cases will be unexpected. Throughout the thrombocytopenic period, the infant is at risk of hemorrhage. The treatment of choice is the transfusion of platelets compatible with the maternal alloantibody. The most reliable donor is the mother. The maternal platelets must be washed, to remove the maternal plasma containing the antibody, and irradiated to eliminate the risk of graft-vs.-host-disease. In an emergency, exchange-transfusion, which removes a part of the circulating antibody, can be considered[43]. IVIgG can be considered, only when hemorrhages are not obvious, because the effect is delayed for 12–18 hours after injection[44].

The current management of subsequent pregnancies after a previous affected child is aimed at preventing ICH during pregnancy and delivery. The optimal antenatal therapy to reverse fetal thrombocytopenia is still a matter of debate[45]. At present, alternatives include weekly maternal injection of high doses of immunoglobulins (IVIgG), with or without corticosteroids, or weekly intrauterine platelet transfusions with antigen-negative platelets. The results obtained by the different teams in Europe and in the USA are somehow different and do not rely on randomized studies[46]. Thus, definite recommendations cannot be provided. Based on our data we recommend the following approach, high-risk pregnant women must be followed in a referral centre. In case of heterozygosity of the father for the offending antigen, fetal platelet genotyping must be performed on amniotic cells[47]. In case of incompatible fetus, a fetal blood sampling should be performed in order to determine the fetal platelet count at around 20–22 weeks of gestation. If the fetus is thrombocytopenic (platelet count $<150 \times 10^9$/l), maternal therapy with IVIgG (1 g/kg/week) should be considered. To assess the efficacy of therapy, a second fetal blood sampling should be performed at around 34 weeks of gestation. Corticosteroids could be added in case of therapy failure. For delivery we propose two options: cesarean section without control of the fetal platelet count or decided following the results of a third fetal blood sampling. In this last case vaginal delivery is allowed if the fetal platelet count is $>50 \times 10^9$/l after maternal therapy or after in utero platelet transfusion. We do not recommend weekly in utero platelet transfusion which seems to be too invasive, and may lead to destruction of the transfused platelets by maternal HLA-alloantibodies, or only in very severe familial cases, when the previous pregnancy was a therapeutic failure with in utero death or intracranial hemorrhage before 20 weeks of gestation.

In any case, pregnant women must be advised to avoid vigorous exercise, trauma, ingestion of drugs interfering with the platelet function (aspirin, antibiotics).

We also recommend the screening of the sisters of the affected woman in order to detect at risk individuals.

Non-immune processes

Infections

Bacterial and non-bacterial infection in the neonate

Thrombocytopenia in bacterial infection of maternal origin is usually moderate, transient and delayed making uncertain its usefulness in the diagnostic investigation. It is often the result of several mechanisms which interact.

Non-bacterial infections in the neonatal period are mostly due to the TORCH agents (Toxoplasmosis, other, rubella, CMV, herpes). Most of them occur after primary infestation during the first months of pregnancy. The typical clinical presentation involves intra uterine growth retardation and jaundice with hepatosplenomegaly. These symptoms prompt the clinician to look for other signs of fetal infection: hemolytic anemia or lesions of bone, eye, heart or cerebrum.

When viral or fungal infections are acquired during the birth process, clinical signs are delayed and depend on the organ affected (myocarditis, encephalitis). Moderate thrombocytopenia is often present at an early stage.

HIV-related thrombocytopenia has been described in infants as early as 3 months old, and has also been reported in neonates.

Neonatal malaria, whether it is a maternofetal or a transfusion-related infection, can lead to thrombocytopenia, probably due in part to low grade DIC.

Viral and bacterial infections in childhood

Viral infections are often associated with alterations of blood cells. Several mechanisms may produce thrombocytopenia[15]. Whereas data argue in favour of direct interactions between viruses and progenitor or peripheral blood cells, other findings support the direct effect of viral-induced autoantibodies on these cells. There are several reports concerning thrombocytopenia after the administration of live measles vaccine[48,49]. Thrombocytopenia has been observed in congenital and acquired rubella[50]. The thrombocytopenia occasionally associated with varicella typically develops 1 to 2 weeks after the rash[50]. Epstein–Barr virus, Cytomegalovirus and Human Parvovirus B19 infections[51] have been associated with thrombocytopenia. Thrombocytopenia is not a rare event in the natural history of HIV infection[52,53]. Thus in the presence of thrombocytopenia, especially the acute manifestations in childhood, it is important to look for viral infection.

Cambridge Books Online © Cambridge University Press, 2009

Thrombocytopenia can be associated with bacterial infection, especially with septicemia.

Increased platelet consumption

Disseminated intravascular coagulation (DIC)
It is frequently observed in distressed neonates or ill children. It usually leads to platelet consumption, typically associated with a decrease in coagulation factors, mostly factor V and fibrinogen, and to the presence of fibrin(ogen) degradation products. Repeated assessment of platelet counts and of coagulation status is mandatory. Usually thrombocytopenia occurs after the other biological signs and can persist a few days after coagulation factors have recovered normal values. The treatment of DIC-related thrombocytopenia relies on the treatment of the underlying cause of DIC itself and of associated symptoms such as hypovolemia, acidosis or shock. However, when the coincidence of coagulation factor consumption and severe thrombocytopenia leads to threatening hemorrhage, the most appropriate treatment has to be considered. Although rare during childhood, such pictures may be observed within the course of acute leukemias, specially at the initiation of the chemotherapy, due to the extensive lysis of hemoblastes, in cases of Kasabach–Merritt syndrome associated with giant hemangiomas, or in newborns who experienced acute, severe fetal distress. In such cases, thrombocytopenia is usually severe, and may account for the bleeding severity. A specific replacement therapy should therefore be undertaken in such cases, at the same time as the specific treatment directed against the DIC underlying pathology. This replacement therapy includes platelet concentrate transfusions or exchange transfusions with blood and platelet concentrates in neonates, transfusion of the missing coagulation factors, generally present in fresh frozen plasma together with coagulation inhibitors, and infusion of antiplasmin inhibitors such as aprotinin, aimed at replacing the alpha-2 antiplasmin consumed within the DIC process[54]. Prothrombin complex concentrates are contra-indicated in such cases as they may aggravate the DIC process. Furthermore, they are of no help in increasing the levels of FV, FVIII and fibrinogen that are consumed during the process.

Among the different causes of DIC, respiratory distress syndrome and perinatal asphyxia in neonates, leukemia or cancer, or bacterial infection, are pre-eminent.

Hemolytic uremic syndrome and thrombotic thrombocytopenic purpura
These two syndromes are characterized by common biological findings: microangiopathy with hemolytic anemia related to red cell fragmentation associated to variable degrees of thrombocytopenia.

The hemolytic uremic syndrome (HUS) described in 1955 could be observed in children[55] in association with a number of different infectious agents in the classic epidemic form. Sporadic and hereditary forms have also been described. Clinical manifestations, laboratory findings and therapy are quite similar to the adult forms. In contrast, thrombotic thrombocytopenic purpura also known as Moschcowitz disease[56] is essentially seen in adults.

Kasabach–Merritt syndrome
This syndrome was reported as the association of hemangioma and thrombocytopenia.

A local platelet consumption occurring locally in the hemangioma may lead to severe thrombocytopenia. Thrombocytopenia is associated with hypofibrinogenemia and red cell fragmentation, which can be seen on blood smears. Overt hemolysis can also be present. The diagnosis may be obvious if the angioma is apparent, but in other cases extensive X-ray and tomography must confirm it. Angioma-related thrombocytopenia can lead to a threatening hemorrhagic syndrome where specific intervention such as vascular embolization is needed. Recently, interferon alpha therapy, which has been shown to inhibit angiogenesis in vitro, has been successfully used in some cases of Kasabach–Merritt Syndrome[57].

Miscellaneous causes
(i) In the course of an extensive thrombosis, such as renal venous or catheter thrombosis, thrombocytopenia can be one of the most relevant biological signs.
(ii) Constitutional severe defects of coagulation inhibitors protein C and S have also to be considered: they may manifest as early as the first day of age by a life-threatening syndrome characterized by extensive necrotic hematomas purpura-fulminans-like, associated with retinal and cerebral thrombosis. Thrombocytopenia is usually severe, associated with schistocytosis and DIC. Due to the low physiological levels of these inhibitors at birth, and to their consumption in the DIC process, the diagnosis has to be confirmed by performing coagulation studies in the parents and siblings and by molecular analysis of the gene. However, due to the dramatic spontaneous aggravation in these cases, a replacement therapy must be initiated in emergency, without waiting for the results.
(iii) Platelet aggregation in the lung leading to thrombocytopenia has been suggested during the course of perinatal aspiration syndrome. A small amount of inhaled amniotic fluid may be a precipitating factor for

Cambridge Books Online © Cambridge University Press, 2009

formation of pulmonary microthrombi, leading to unresponsive persistent pulmonary hypertension.

Miscellaneous causes of acquired thrombocytopenia

In sick children, thrombocytopenia is more often easily related to causes extensively reviewed by Beardsley[58].

(i) Heparin-related thrombocytopenia has been reported in young infants, but not in the newborn. However, the potential hazards of the thrombocytopenia induced by platelet aggregation must be kept in mind, whatever the age at which heparin is prescribed, and must prompt discontinuation of therapy.

(ii) Thrombocytopenia has also been reported in infants who have experienced cold injury. It could be the combined result of a platelet splenic sequestration and of a decrease in megakaryocytopoiesis. Both mechanisms have been observed in hibernating animal models. The former has been reported in humans undergoing hypothermia for surgery and the latter occurs after a long exposure.

(iii) Neonatal thrombocytopenia is frequently observed during the first postnatal days of very small-for-date babies or in babies from pre-eclamptic mothers, often associated with erythroblastosis and granulocytopenia. In the most severe cases, hepatic insufficiency and multiorgan failure accompany neonatal thrombocytopenia, which can be fatal. Chronic hypoxia is likely to explain neonatal thrombocytopenia in these cases for it has been shown, in animal models, that prolonged hypoxia in pregnant females could lead to fetal thrombocytopenia[59]. This process could explain most cases of neonatal thrombocytopenia observed in neonatal intensive care units.

In a great number of other cases, peripheral destruction and impairment of megakaryocytopoiesis are combined, and there is no way to discriminate their respective involvement.

Neonatal thrombocytopenia has been observed in infants with inherited metabolic disorders leading to acidosis and after several therapeutic procedures such as phototherapy, which has been shown to shorten platelet life span, or exchange transfusion, where the magnitude of the transient fall observed during 2–3 days after the procedure could depend on the storage conditions of the blood used. Neonatal thrombocytopenia related to antenatal maternal treatment with agents such as thiazide and tolbutamide has been reported, but should be diagnosed with caution.

Inherited thrombocytopenias

Although its real incidence remains unknown, constitutional thrombocytopenia is not a rare event. The inherited origin of thrombocytopenia has to be systematically considered in children before the initiation of potentially harmful treatments, especially when thrombocytopenia is long lasting, moderate and silent and refractory to conventional AITP therapy. Achieving the diagnosis can need a careful collection of medical and familial data in search for consanguinity, thrombocytopenia or bleeding history in family member(s). A detailed pedigree could establish the inheritance pattern. Platelet abnormalities on blood smears can be observed as small, giant or agranular platelets. Other associated symptoms must be carefully searched for, such as immunodeficiency in Wiskott–Aldrich syndrome, deafness, nephritis, cataract or abnormalities in other hematopoietic cell lineages, e.g. presence of Döhle bodies in the neutrophils. Platelet functions must be assessed in order to detect associated thrombopathies.

Inherited thrombocytopenias can be classified according to the platelet size (Table 37.5).

Small platelet size

A small platelet size is the hallmark of Wiskott–Aldrich syndrome (WAS). It is a severe X-linked thrombocytopenia, associated with immunodeficiency and eczema. It has recently been related to mutations of the gene encoding WAS protein (WASP)[60], which is present in neutrophils, mononuclear cells and platelets, and mapping at Xp11.22–11.23. Thrombocytopenia and lymphocyte defects are severe in most children after the age of 6. Bleeding remains the major cause of death in these patients and may be alleviated by splenectomy in some cases. Bone marrow transplantation is the actual therapeutic option in severe cases with repeated infection. A mechanism has recently been proposed for the thrombocytopenia and small platelet size, which is based on the increased Ca^{2+} level in platelets, resulting in platelet activation, phosphatidylserine exposure at the platelet surface resulting in increased recognition and phagocytosis by the spleen macrophages, and microparticle release, leading to small platelet size[61].

Mutations of the WASP locus have also been reported in atypical WAS or in isolated X-linked thrombocytopenias (XLT) without immunodeficiency[62,63]. XLT may be misdiagnosed as chronic autoimmune thrombocytopenic purpura.

Cambridge Books Online © Cambridge University Press, 2009

Table 37.5. Inherited thrombocytopenias

Small platelet size
Wiskott–Aldrich syndrome: X-linked thrombocytopenia associated with immunodeficiency and eczema
Isolated X-linked thrombocytopenia without immunodeficiency
Inherited giant platelet disorders
Autosomal dominant inheritance
May-Hegglin disease, Sebastian syndrome: basophilic inclusions in neutrophils (Döhle inclusions)
Alport syndrome: deafness, and nephritis
Epstein syndrome: Alport syndrome with severe thrombocytopenia
Fechtner syndrome: deafness, nephritis and congenital cataract with Döhle inclusions in neutrophils
Recessive disorder
Bernard–Soulier syndrome: defect in the platelet glycoprotein complex Ib–IX–V
Velocardiofacial (Digeorge) syndrome: heterozygous variant of Bernard–Soulier syndrome
Platelet-type von Willebrand syndrome: anomaly of the glycoprotein Ibα
Grey platelet syndrome: lack of alpha granules
Normal platelet volume
TAR syndrome: thrombocytopenia with absent radii, defective signal transduction in the c-Mpl pathway, autosomal recessive inheritance
Congenital amegakaryocytic thrombocytopenia (CAMT): Mpl mutations
Paris–Trousseau thrombocytopenia: giant alpha granules, 11q23.3 deletion, autosomal dominant inheritance

Inherited giant platelet disorders

A large platelet size is observed in inherited giant platelet disorders, recently reviewed[64]. Some are associated with basophilic inclusions in the neutrophils called Döhle bodies in May–Hegglin disease, and slightly different inclusions in Sebastian syndrome. Although thrombocytopenia may be severe, the low number of platelets seems to be balanced by their large size leading to a near-normal thrombocrit, and to a moderate bleeding tendency, occurring mostly after trauma and surgery. Their inheritance is autosomal dominant and thrombocytopenia has been suggested to result from an impaired megakaryocyte fragmentation. The May–Hegglin molecular anomaly has recently been located on MYH9, a region of chromosome 22q12–13 encoding non-muscle myosin heavy chain A. Mutations of this gene have been identified in 30 affected members of five unrelated families, whereas it was not found in 40 normal individuals[65].

(i) Giant thrombocytopenia associated with systemic manifestations, of autosomal dominant inheritance, characterizes Alport syndrome (deafness and nephritis), Epstein syndrome (Alport syndrome with severe thrombocytopenia), Fechtner syndrome (deafness, nephritis and congenital cataract, also associated with Döhle inclusions). Very little is known about the pathogenesis of these disorders, of which the molecular anomalies still have to be identified.

(ii) Giant thrombocytopenias associated with ultrastructural defects are better characterized:

Bernard–Soulier syndrome (BSS) is an autosomal recessive disorder due to a defect in the platelet glycoprotein (GP) Ib–IX–V complex, which is the receptor for von Willebrand factor (vWF) involved in platelet adhesion to the subendothelium. In most cases, a mutation of one of the genes coding for the GPIb α or β chains or GPIX leads to an abnormal assembly of the GP complex resulting in an impaired platelet adhesion. The clinical expression varies from mild to severe. Bleeding time may be normal in mild cases. Platelet half-life is decreased. Megakaryocytes may be abnormal, displaying vacuoles and abnormal demarcation membranes in ultrastructural study. The diagnosis relies on the absence of ristocetin induced platelet agglutination and on a decreased or undetectable GPIb–IX complex evidenced by flow cytometric analysis of the platelet glycoproteins[66].

(iii) Giant platelet with velocardiofacial (Digeorge) syndrome is an autosomal recessive disease. It is due to a 22q11 deletion including a GPIbβ gene deletion, and it is therefore considered a heterozygous variant of BSS.

(iv) Platelet-type von Willebrand syndrome, due to an anomaly of the GPIbα, is the platelet counterpart of the von Willebrand type 2B plasmatic disease, where the molecular anomaly is located on exon 28 of the vWF gene, encoding the vWF binding domain to GPIbα. Both anomalies induce an increased binding of vWF to its receptor, resulting in a decrease of circulating high molecular weight multimers of vWF and a bleeding tendency. The diagnosis relies on a highly fluctuating platelet count, ranging in the same individual from severe thrombocytopenia to normal platelet counts, an increased ristocetin-induced platelet agglutination, a decreased circulating vWF ristocetin cofactor activity, and the identification of the gene defect. Platelet ultrastructural study performed in three unrelated patients showed that vWF association to GPIb on the platelet surface and also inside the surface of the

Cambridge Books Online © Cambridge University Press, 2009

connected canalicular system does not result in platelet activation, which could provide an explanation for the bleeding tendency observed in these diseases. Furthermore, since GPIb integrity has been shown to be required for the normal platelet release from megakaryocyte proplatelet processes, abnormal occupancy of GPIb by vWF might interfere with platelet production, and account for thrombocytopenia[67].

Grey platelet syndrome is an autosomal recessive disease associating a moderate thrombocytopenia and a lack of alpha granules. Myelofibrosis is often observed, due to an abnormal release of platelet derived growth factor (PDGF) normally located inside the granules. The diagnosis relies on the platelet aspect on the blood smears and on a defective collagen induced platelet aggregation and secretion.

Other thrombocytopenias with giant platelets such as Mediterranean thrombocytopenia, resulting in moderate or no bleeding, are classified as 'benign'.

Normal platelet volume

Thrombocytopenia with absent radii or TAR syndrome is a rare, autosomal recessive disease characterized by a severe amegakaryocytic thrombocytopenia, associated with skeletal abnormalities such as radial aplasia, and a polymalformative syndrome extensively described by Hedberg[68]. Normal expression of c-Mpl on platelets with defective signal transduction in the c-Mpl pathway has been observed[69]. The platelet count may increase during the first months of life. On the other hand, the two main features of this rare syndrome may be isolated. Neonatal thrombocytopenia is probably the result of an arrest in the development of committed megakaryocyte progenitor cells, whereas production of megakaryocyte growth factors is normal. In early infancy thrombocytopenia is usually symptomatic, with life-threatening bleeding episodes. It has been shown that some megakaryocytic progenitors could respond to PEG-rHuMGDF, but this response could be incomplete.

Congenital amegakaryocytic thrombocytopenia (CAMT) has recently been shown to be due to Mpl mutations[70].

Paris–Trousseau is an autosomal dominant thrombocytopenia associated with a mild hemorrhagic presentation due to thrombopathy, and characterized by the presence of giant alpha-granules. There is an impaired megakaryocytopoiesis secondary to a 11q23.3 deletion[71].

During the last few years, many molecular abnormalities have been described, allowing secure diagnosis of constitutional thrombocytopenia. Investigations required for the diagnosis of inherited thrombocytopenia should be systematically performed in cases of non-acute thrombocytopenias. Thanks to the automated blood cell counters, the platelet count is nowadays systematically assessed together with leukocytes and erythrocytes, leading to the disclosure of silent, asymptomatic thrombocytopenias.

References

1 Berkman N, Michaeli Y, Or R, Eldor A. EDTA-dependent pseudothrombocytopenia: a clinical study of 18 patients and a review of the literature. *Am J Hematol* 1991; 36: 195–201.
2 Sola MC, Juul SE, Meng YG et al. Thrombopoietin (Tpo) in the fetus and neonate: Tpo concentrations in preterm and term neonates, and organ distribution of Tpo and its receptor (c-mpl) during human fetal development. *Early Hum Dev* 1999; 53: 239–250.
3 Sola MC, Calhoun DA, Hutson AD, Christensen RD. Plasma thrombopoietin concentrations in thrombocytopenic and non-thrombocytopenic patients in a neonatal intensive care unit. *Br J Haematol* 1999; 104: 90–92.
4 Watts TL, Murray NA, Roberts IA. Thrombopoietin has a primary role in the regulation of platelet production in preterm babies. *Pediatr Res* 1999; 46: 28–32.
5 McMillan R, Tani P, Millard F. Platelet-associated and plasma anti-glycoprotein autoantibodies in chronic ITP. *Blood* 1987; 70: 1040–1045.
6 Kiefel V, Santoso S, Weisheit M, Mueller-Eckhardt C. Monoclonal antibody-specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet-reactive antibodies. *Blood* 1987; 70: 1722–1726.
7 Brighton TA, Evans S, Castaldi PA, Chesterman CN, Chong BH. Prospective evaluation of the clinical usefulness of an antigen-specific assay (MAIPA) in idiopathic thrombocytopenic purpura and other immune thrombocytopenias. *Blood* 1996; 88: 194–201.
8 Berchtold P, Imbach P, Muller D et al. Results of the first international standardization workshop on platelet-associated and circulating plasma autoantibodies in autoimmune thrombocytopenia (AITP). *Blood* 1994; 84: 186. [Abstract].
9 Ishiguro A, Nakahata T, Matsubara K, Hayashi Y, Kato T, Suzuki Y. Age-related changes in thrombopoietin in children: reference interval for serum thrombopoietin levels. *Br J Haematol* 1999; 106: 884–888.
10 Gill KK, Kelton JG. Management of idiopathic thrombocytopenic purpura in pregnancy. *Semin Hematol* 2000; 37: 275–289.
11 Valat AS, Caulier MT, Devos P et al. Relationships between severe neonatal thrombocytopenia and maternal characteristics in pregnancies associated with autoimmune thrombocytopenia. *Br J Haematol* 1998; 103: 397–401.
12 Gandemer V, Kaplan C, Quelvennec E et al. Pregnancy-associated autoimmune neonatal thrombocytopenia: role of maternal HLA genotype. *Br J Haematol* 1999; 104: 878–885.
13 Kaplan C, Daffos F, Forestier F et al. Fetal platelet counts in thrombocytopenic pregnancy. *Lancet* 1990; 336: 979–982.

Cambridge Books Online © Cambridge University Press, 2009

14 Zeller B, Helgestad J, Hellebostad M et al. Immune thrombocytopenic purpura in childhood in Norway: a prospect registration. *Pediat Hematol and Oncol* 2000; 17: 551–558.
15 Rand ML, Wright JF. Virus-associated idiopathic thrombocytopenic purpura. *Transfus Sci* 1998; 19: 253–259.
16 George JN, Woolf SH, Raskob GE et al. Idiopathic thrombocytopenic purpura: A guideline for diagnosis and management in children and adults. *Ann Med* 1998; 30: 38–44.
17 Blanchette V, Carcao M. Approach to the investigation and management of immune thrombocytopenic purpura in children. *Semin Hematol* 2000; 37: 299–314.
18 Lilleyman JS. Chronic childhood idiopathic thrombocytopenic purpura. Baillière's *Clin Haematol* 2000; 13: 469–483.
19 Robb LG, Tiedeman K. Idiopathic thrombocytopenic purpura: predictors of chronic disease. *Arch Dis Child* 1990; 65: 502–506.
20 Lilleyman JS. Management of childhood idiopathic thrombocytopenic purpura. *Br J Haematol* 1999; 105: 871–875.
21 Reid MM. Chronic idiopathic thrombocytopenic purpura: incidence, treatment and outcome. *Arch Dis Child* 1994; 72: 125–128.
22 Vesely S, Buchanan GR, Cohen A, Raskob G, George J. Self-reported diagnostic and management strategies in childhood idiopathic thrombocytopenic purpura: results of a survey of practicing pediatric hematology/oncology specialists. *J Pediatr Hematol Oncol* 2000; 22: 55–61.
23 Eden OB, Lilleyman JS, on behalf of the British Paediatric Haematology Group. Guidelines for management of idiopathic thrombocytopenic purpura. *Arch Dis Child* 1992; 67: 1056–1058.
24 George JN, Woolf SH, Raskob GE et al. Idiopathic thrombocytopenic purpura: a practice guideline developed by explicit methods for the American Society of Hematology. *Blood* 1996; 88(1): 3–40.
25 Buchanan GR, de Alarcon PA, Feig SA et al. Acute idiopathic thrombocytopenic purpura-Management in childhood. *Blood* 1997; 89: 1464–1465.
26 Blanchette V, Imbach P, Andrew M et al. Randomised trial of intravenous immunoglobulin G, intravenous anti-D, and oral prednisone in childhood acute immune thrombocytopenic purpura. *Lancet* 1994; 344: 703–707.
27 Buchanan GR. Childhood acute idiopathic thrombocytopenic purpura: how many tests and how much treatment required? *J Pediatr* 1985; 106: 928–930.
28 Carcao MD, Zipursky A, Butchatt S et al. Short-course oral prednisone therapy in children presenting with acute immune thrombocytopenic purpura. *Acta Paediatr* 1998; 87: 71–74.
29 Imbach P, d'Apozzo V, Hirt A et al. High-dose intravenous gammaglobulin for idiopathic thrombocytopenic purpura in childhood. *Lancet* 1981; i: 1228–1230.
30 Imbach P, Barandun S, Hirt A, Wagner HP. Intravenous immunoglobulin for idiopathic thrombocytopenic purpura (ITP) in childhood. *Am J Pediatr Hematol Oncol* 1984; 6: 171–174.
31 Ahsan N. Intravenous immunoglobulin induced-nephropathy: a complication of IVIG therapy. *J Nephrol* 1998; 11: 157–161.
32 Kenny-Walsh E. Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin Irish Hepatology Research Group. *N Engl J Med* 1999; 340: 1228–1233.
33 Young GMD, Luban N, White JG. Giant platelet disorders in African–American children misdiagnosed as idiopathic thrombocytopenic purpura. *J Pediatr Hematol Oncol* 1999; 21: 231–236.
34 Siegel RS, Rae JL, Barth S et al. Platelet survival and turnover: important factors in predicting response to splenectomy in immune thrombocytopenic purpura. *Am J Hematol* 1989; 30: 206–212.
35 Walker RW, Walker W. Idiopathic thrombocytopenia, initial illness and long term follow up. *Arch Dis Child* 1984; 59: 316–322.
36 Scaradavou A, Woo B, Woloski BMR et al. Intravenous anti-D treatment of immune thrombocytopenic purpura: Experience in 272 patients. *Blood* 1997; 89: 2689–2700.
37 Rosse WF. Whatever happened to vinca-loaded platelets? *N Engl J Med* 1984; 310: 1051–1052.
38 Durand-Zaleski I, Schlegel N, Blum-Boisgard C et al. Screening primiparous women and newborns for fetal/neonatal alloimmune thrombocytopenia: A prospective comparison of effectiveness and costs. *Am J Perinatol* 1996; 13: 423–431.
39 Dreyfus M, Kaplan C, Verdy E et al. Frequency of immune thrombocytopenia in newborns: a prospective study. *Blood* 1997; 89: 4402–4406.
40 Mueller-Eckhardt C, Kiefel V, Grubert A et al. 348 cases of suspected neonatal allo-immune thrombocytopenia. *Lancet* 1989; i: 363–366.
41 Kaplan C, Morel-Kopp MC, Clemenceau S, Daffos F, Forestier F, Tchernia G. Fetal and neonatal alloimmune thrombocytopenia: current trends in diagnosis and therapy. *Transfus Med* 1992; 2: 265–271.
42 Kroll H, Kiefel V, Santoso S. Clinical aspects and typing of platelet alloantigens. *Vox Sang* 1998; 74 (Suppl.2): 345–354.
43 Bussel JB, Kaplan C, McFarland JG, and the Working Party on Neonatal Immune Thrombocytopenia of the Neonatal Hemostasis Subcommittee of the Scientific and Standardization Committee of the ISTH. Recommendations for the evaluation and treatment of neonatal autoimmune and alloimmune thrombocytopenia. *Thromb Haemost* 1991; 65: 631–634.
44 Massey GV, McWilliams NB, Mueller DG, Napolitano A, Maurer HM. Intravenous immunoglobulin in treatment of neonatal isoimmune thrombocytopenia. *J Pediatr* 1987; 111: 133–135.
45 Kaplan C, Murphy MF, Kroll H, Waters AH. Feto-maternal alloimmune thrombocytopenia: antenatal therapy with IvIgG and steroids – more questions than answers. *Br J Haematol* 1998; 100: 62–65.
46 Bussel J, Kaplan C. The fetal and neonatal consequences of maternal alloimmune thrombocytopenia. In: Michiels JJ, ed. *Acquired disorders of haemostasis: pathophysiology, clinical practice and basic research*. London: Bailliere Tindall, 1998: 391–408.
47 Clemenceau S, Perichon B, Elion J, Kaplan C, Krishnamoorthy

Cambridge Books Online © Cambridge University Press, 2009

R. An improved DNA-based identification of fetuses at risk for HPA-1a (PlA1) neonatal alloimmune thrombocytopenia. *Br J Haematol* 1994; 86: 198–200.

48 Jonville-Bera AP, Autret E, Galy-Eyraud C, Hessel L. Thrombocytopenic purpura after measles, mumps and rubella vaccination: a retrospective survey by the French regional pharmacovigilance centres. *Pediatr Infect Dis* 1981; 15: 44–48.

49 Kiefaber RW. Thrombocytopenic purpura after measles vaccination. *N Engl J Med* 1981; 305: 225–236.

50 Kahane S, Dvilansky A, Estok L. Detection of anti-platelet antibodies in patients with idiopathic thrombocytopenic purpura (ITP) and in patients with rubella and herpes group viral infections. *Clin Exp Immunol* 1981; 44: 49–56.

51 Heegaard ED, Rosthoj S, Petersen BL, Nielsen S, Karup Pedersen F. Role of parvovirus B19 infection in childhood idiopathic thrombocytopenic purpura. *Acta Paediatr* 1999; 88: 614–617.

52 Monpoux F, Kurzenne JY, Sirvent N, Cottalorda J, Boutte P. Partial splenectomy in a child with human immunodeficiency virus-related immune thrombocytopenia. *J Pediatr Hematol Oncol* 1999; 21: 441–443.

53 Young G, Loechelt BJ, Rakusan TA, Nichol JL, Luban NL. Thrombopoietin levels in HIV-associated thrombocytopenia in children. *J Pediatr* 1998; 133: 765–769.

54 Avvisati G, Büller HR, Ten Cate JW, Mandell F. Tranexamic acid for control of hemorrhage in acute promyelocytic leukemia. *Lancet* 1989; 338: 122–124.

55 Renaud C, Niaudet P, Gagnadoux MF, Broyer M, Habib R. Haemolytic uraemic syndrome: prognostic factors in children over 3 years of age. *Pediatr Nephrol* 1995; 9: 24–29.

56 Marcus AJ. Moschcowitz revisited. *N Engl J Med* 1982; 307: 1447–1448.

57 White CW. Treatment of hemangiomatosis with recombinant interferon alpha. *Semin Hematol* 1990; 27(3, suppl 4): 15–22.

58 Beardsley DS, Nathan DG. Platelet abnormalities in infancy and childhood. In: Nathan DG, Orkin SH, eds. *Hematology of infancy and childhood*; New York: WB Saunders; 1997: 1585–1630.

59 Meberg A. Transitory thrombocytopenia in newborn mice after intrauterine hypoxia. *Pediatr Res* 1980; 14: 1071–1073.

60 Derry JMJ, Ochs HJ, Francke U. Isolation of a novel gene mutated in Wiskott–Aldrich syndrome. *Cell* 1994; 78: 635–644.

61 Shcherbina A, Rosen FR, Remold-O'Donnel E. Pathological events in platelets of Wiskott–Aldrich syndrome patients. *Br J Haematol* 1999; 106: 875–883.

62 Villa A, Notarangelo L, Macchi P et al. X-linked thrombocytopenia and Wiskott–Aldrich syndrome are allelic diseases with mutations in the WASP gene. *Nat Genet* 1995; 9: 414–417.

63 Zhu Q, Zhang M, Blaese RM et al. The Wiscott–Aldrich syndrome and X-linked congenital thrombocytopenia are caused by mutation of the same gene. *Blood* 1995; 86: 3797–3804.

64 Mhawech P, Saleem A. Inherited giant platelet disorders; classification and literature review. *Am J Clin Pathol* 2000; 113: 176–190.

65 Kelley MJ, Jawien W, Ortel TL, Korczak JF. Mutation of MYH9, encoding non-muscle myosin heavy chain A in May-Hegglin anomaly. *Nature Genet* 2000; 26: 106–108.

66 Nurden A. Inherited abnormalities of platelets. *Thromb Haemost* 1999; 82: 468–480.

67 Nurden P, Chretien F, Poujol C, Winckler J, Borel-Derlon A, Nurden A. Platelet ultrastructural abnormalities in three patients with type 2B von Willebrand disease. *Br J Haematol* 2000; 110: 704–714.

68 Hedberg VA, Lipton JM. Thrombocytopenia with absent radii: a review of 100 cases. *Am J Pediatr Hematol Oncol* 1988; 10: 51–64.

69 Ballmaier M, Schulze H, Strauss G. Thrombopoietin in patients with congenital thrombocytopenia and absent radii: elevated serum levels, normal receptor expression, but defective reactivity to thrombopoietin. *Blood* 1997; 90: 612–619.

70 van den Oudenrijn S, Bruin M, Folman CC et al. Mutations in the thrombopoietin receptor, Mpl, in children with congenital amegakaryocytic thrombocytopenia. *Br J Haematol* 2000; 110: 441–448.

71 Breton-Gorius J, Favier R, Guichard J et al. A new congenital dysmegakaryopoietic thrombocytopenia (Paris-Trousseau) associated with giant platelet alpha-granules and chromosome 11 deletion at 11q23. *Blood* 1995; 85: 1805–1814.

Cambridge Books Online © Cambridge University Press, 2009

Alloimmune thrombocytopenia

E. Anders Kolb and James B. Bussel

Cornell University Medical Centre, New York Hospital, USA

Fetal and neonatal alloimmune thrombocytopenia (AIT) is the most common cause of severe thrombocytopenia in fetuses and neonates[1]. Maternal IgG alloantibodies against paternally derived fetal platelet antigens cross the placenta early in pregnancy and commonly result in severe thrombocytopenia. While the reported incidence varies somewhat with the assigned threshold of thrombocytopenia (50, 100, or 150×10^9/l), in most unselected populations, AIT affects 1 in 1000 to 1 in 2000 live births. Table 38.1 contains recent studies of AIT in unselected populations, systematically screened. In its severe form, AIT has the potential for significant morbidity (including intracranial hemorrhage) and mortality. In milder forms, there are either antibodies with no thrombocytopenia, or mild to moderate thrombocytopenia, which is identified only by a complete blood count obtained for another indication or in a screening study. While there have been extensive efforts made in the diagnosis and characterization of the disease, strategies for early detection and intervention remain controversial.

Pathogenesis

There are three requisite components of the pathogenesis of AIT. First, there must be an incompatibility between maternal and fetal 'platelet-specific' antigens, which are inherited from the father. The second requirement, alloimmunization, is a maternal humoral immune response specific to these 'foreign' fetal platelet antigens. Finally, maternal anti-platelet IgG alloantibodies must cross the placenta, bind to fetal platelet antigens, and cause fetal platelet destruction with resultant thrombocytopenia. The term alloimmunization, therefore, implies that there is a parental antigen incompatibility, and that maternal antibodies are produced and are specific to the paternally inherited (and foreign to the mother) fetal platelet antigen. The predictability of this process, and its ability to cause fetal or neonatal thrombocytopenia, is well characterized serologically but perplexing in a number of areas, and warrants careful discussion.

Table 38.1. Incidence of neonatal thrombocytopenia

Reference	Number and population screened	Incidence of AIT	Comments
	Pregnant women		
14	24 417	1 in 1285 pregnancies	
	Neonates		
1	15 932	1 in 5000 newborns	Platelet count $<50 \times 10^9$/l*
40	5 632	1 in 1000 newborns	
13	9 142	1 in 1800 newborns	Platelet count $<100 \times 10^9$/l
46	8 388	1 in 1700 newborns	
42	4 489	1 in 1200 newborns	
16	24 101	1 in 2400 newborns	Platelet count $<100 \times 10^9$/l
27	5 632	1 in 1000 newborns	

Notes:
* Unless otherwise specified, neonatal thrombocytopenia is defined as a cord blood platelet count less than 150×10^9/l.

Table 38.2. Human Platelet Antigen Nomenclature and Frequency

HPA Name	Other names	Glycoprotein	DNA allele (amino acid change)	Gene frequency (Caucasian)	Serologic frequency	
					White	Japanese
HPA-1a	Zw^{a}, PL^{A1}	GPIIIa	Leu$_{33}$Arg$_{143}$Pro$_{407}$Arg$_{489}$Arg$_{636}$	0.85	97.9%	99.9%
HPA-1b	Zw^{b}, PL^{A2}	GPIIIa	Pro$_{33}$Arg$_{143}$Pro$_{407}$Arg$_{489}$Arg$_{636}$	0.15	26.5%	3.7%
HPA-2a	Ko^{b}	GPIb	Thr$_{145}$	0.93	99.3%	NT*
HPA-2b	Ko^{a}, Sib^{a}	GPIb	Met$_{145}$	0.07	14.6%	35.4%
HPA-3a	Bak^{a}, Luk^{a}	GPIb	Ile$_{843}$	0.61	87.7%	78.9%
HPA-3b	Bak^{b}	GPIIb	Ser$_{843}$	0.39	64.1%	NT
HPA-4a	Pen^{a}, Yuk^{b}	GPIIIa	Leu$_{33}$Arg$_{143}$Pro$_{407}$Arg$_{489}$Arg$_{636}$	0.85	99.9%	99.9%
HPA-4b	Pen^{b}, Yuk^{a}	GPIIIa	Leu$_{33}$Gln$_{143}$Pro$_{407}$Arg$_{489}$Arg$_{636}$	<0.01	0.2%	1.7%
HPA-5a	Br^{b}, Zav^{b}	GPIa	Glu$_{505}$	0.89	99.2%	NT
HPA-5b	Br^{a}, Zav^{a}, Hc^{a}	GPIa	Lys$_{505}$	0.11	20.6%	NT
HPA-6bW**	Ca^{a}, Tu^{a}	GPIIIa	Leu$_{33}$Arg$_{143}$Pro$_{407}$Gln$_{489}$Arg$_{636}$	0.85	?	?
		GPIIIa	Leu$_{33}$Arg$_{143}$Glu$_{407}$Arg$_{489}$Arg$_{636}$	<0.01	?	?
HPA-7bW	Mo^{a}	GPIIIa	Leu$_{33}$Arg$_{143}$Ala$_{407}$Arg$_{489}$Arg$_{636}$	0.85	<1	?
		GPIIIa	Leu$_{33}$Arg$_{143}$Pro$_{407}$Arg$_{489}$Arg$_{636}$	<0.01	?	?
HPA-8bW	Sr^{a}	GPIIIa	Leu$_{33}$Arg$_{143}$Pro$_{407}$Arg$_{489}$Cys$_{636}$	0.85?	?	
		GPIIIa	Leu$_{33}$Arg$_{143}$Pro$_{407}$Arg$_{489}$Arg$_{636}$	<0.01	?	?
HPA-9bW	Max^{a}	GPIIb	Met$_{837}$			
			Val$_{837}$			
HPA-10bW	La^{a}	GPIIIb	Gln$_{62}$			
			Arg$_{62}$			
HPA-11bW	Gro^{a}	GPIIIa	His$_{633}$			
			Arg$_{633}$			
HPA-12bW	Iy^{a}	GPIb	Glu$_{15}$			
			Gly$_{15}$			
HPA-13bW	Sit^{a}	GpIa	Met$_{799}$			
			Thr$_{799}$			
HPA-	Oe^{a}	GPIIIa	dLys$_{611}$			
HPA-	Va^{a}	GPIIIa				
HPA-	Pe^{a}	GP1b$_{\alpha}$				

Notes:
* NT = Not tested.
** 'W' indicates antigens for which only one allele is identified.

Human platelet-specific antigens

Platelets share several antigen systems with other cell types, including the HLA class I antigens, and the ABO blood group antigens[2,3]. However, these antigen systems are rarely, if ever, implicated in AIT. AIT secondary to an HLA antigen mismatch remains unproven and controversial. More commonly, AIT is caused by antibodies to platelet-specific antigens, which represent epitopes of the platelet surface glycoproteins. Currently there are at least fifteen human platelet-specific antigens listed by the Platelet Serology Working Party of the International Society of Blood Transfusion (ISBT), each with a different serologic frequency and ethnic distribution[2,4–6]. In 1990 the ISBT Working Party on Platelet Serology formulated a new nomenclature for the human platelet antigen system, as an attempt to standardize an otherwise confusing system of antigen names and descriptions. Platelet-specific antigens are designated HPA for Human Platelet Antigen. They are numbered in order of their original description, and the alleles labelled alphabetically in the order of their serologic frequency[2,4]. Recently, DNA sequencing has permitted the description of the specific amino acid changes associated with each allele. This DNA-based typing system provides an additional nomenclature for describing human platelet-specific antigens. Each HPA type is biallelic, and autosomal co-dominant, differing in only one amino acid. It is important to note that there is overlap of certain alleles, as discussed by Newman[7], and that the naming of a certain allele implies an understanding of the molecular identity of that glycoprotein. Table 38.2 contains the HPA type, the glycoprotein on which it is located, the previous

Cambridge Books Online © Cambridge University Press, 2009

Table 38.3. Clinically significant AIT

Reference	Patients (number) and entry criteria	Bleeding symptoms	Antigen incompatibility
9	88 Thrombocytopenic[b] infants with AIT	Petechiae (90%) Hematomas (66%) Melena (28%) Intracranial hemorrhage (14%) Hemoptysis (8%) Hematuria (7%) Retinal extravasations (7%) Hematemesis (2%) Death (1%)[a] Platelet count $<30\times10^9$/l (95%)	HPA-1a
12	24 Infants with AIT	Petechiae (58%) Intracranial hemorrhage (21%) 1 patient died as a result of an ICH[c]	HPA-1a
13	46 Thrombocytopenic infants with AIT	Petechiae, purpura (83%) Visceral bleeding (15%) Intracranial hemorrhage (11%) Platelet count $<50\times10^9$/l (87%)	HPA-1a, HPA-1b, and HPA-5b

Notes:
[a] Died at 6 months of age from complications of the ICH.
[b] Thrombocytopenia defined as a platelet count less than 100×10^9/l.
[c] ICH = Intracranial hemorrhage.

antigen designations, the associated amino acid changes for each allele, and the frequency of each antigen in selected populations.

AIT has been reported after alloimmunization to numerous human platelet antigens (see Table 38.3 and 38.4). In almost all large series, HPA1a incompatibility is the most common incompatibility resulting in AIT[8–16]. Newer studies suggest that HPA-5b incompatibility may be more common than HPA-1a incompatibility, but severe thrombocytopenia after HPA-5b alloimmunization is rare[17–19]. Of note, the initial description of a new human platelet antigen is frequently initiated by a patient with significant morbidity. Therefore, most non-HPA-1a incompatibilities have severe index cases, but incidences appear to be very low. Despite numerous reports of new, and potentially severe or frequent incompatibilities, it remains clear that HPA-1a incompatibility remains the most common cause of severe AIT in Caucasian populations.

Alloimmunization

Fetal platelet antigens may be expressed as early as 16 weeks gestation[20], and alloantibody formation with fetal platelet destruction may occur as early as 16 to 20 weeks of gestation[21,22]. Unlike hemolytic disease of the newborn, alloimmunization is reported in up to 50% of first pregnancies. However, antibodies have not been detected in primiparous women prior to 17 weeks gestation[14]. In a large series, Williamson et al.[14] reported that in 46 sensitized HPA-1a negative women, alloantibodies were detected before the 20th week of gestation in 59%, between 21 and 34 weeks in 17%, between 33 weeks and term in 6.5%, and on postnatal testing only in 6.5%.

Alloantibodies to fetal HPA-1a on GPIIIa is the most common cause of severe AIT in Caucasian populations[9]. Van Loghem et al.[23] initially reported that 97% of North American and European Caucasians react to anti-HPA-1a. The Hardy-Weinberg law predicts the allelic frequency to be 0.83 for HPA-1a and 0.17 for HPA-1b. Therefore, in Caucasians of European or North American descent, the genotypic distribution is calculated to be 68.9% homozygosity for HPA-1a, 2.9% homozygosity for HPA-1b, and 28.2% heterozygosity (HPA-1a/HPA-1b)[24,25]. The frequency of the HPA-1b genotype has been confirmed in several large population studies[14,15,26,27]. Both the predicted and actual frequency of mothers at risk for HPA-1a incompatibility, by virtue of being homozygous for HPA-1b, is 2–3%. The HPA-1b homozygous mother will be susceptible to alloimmunization in all cases if her partner is homozygous for HPA-1a (68.9%), or in half the offspring of a heterozygous partner (14.1%). Based on

Cambridge Books Online © Cambridge University Press, 2009

Table 38.4. Less Common Antigen Incompatibilities in AIT

HPA type	Reference	Patients reported	Platelet Nadir ($\times 10^9$cells/l)	Hemorrhagic Sequelae	Maternal HLA Type
HPA-1b	38,115,116,117,118,119	6 total	10–13 duration up to 6 days	Petechiae (2 patients) Schizencephaly (1 patient) Prolonged thrombocytopenia 1 patient)	No consistent association
HPA-2b	42	3[a]	Mild	None	
HPA-3a	120	1	13	Purpura Death from ICH	None reported
HPA-3b	42 65	4[a] 14	Mild <20	None ICH (2 patients)	
HPA-4a	61,62	2 (siblings)	6–12	Petechiae (2 patients) Ecchymoses (2 patients) Scalp Hematoma (1 patient) In utero ICH (2 patients)	No consistent association
HPA-5b	121,17,9,10,13,18,40,46	39	<10 (4 patients) 10–30 (13 patients) >30 (22 patients)	No symptoms (59%) Purpura or hematoma (18%) Visceral hemorrhage (15%) ICH (8%) Death due to ICH (1 patient)	Associated with HLA-DR6
HPA-6b	42,122,123			Mild	Associated with maternal HLA DRB*1501; DQA1*0102; DQB1*0602 (Westerman,1997)

Notes:
[a] AIT suspected due to antigen incompatibility and thrombocytopenia, but there was no antibody detected.

these gene frequencies, alloimmune thrombocytopenia as a result of HPA-1a incompatibility may develop in 1 of 42 pregnancies[25]. The actual incidence, as stated above, is 1 in 1000 to 1 in 2000. Several prospective studies have shown that only 4–14% of mothers homozygous for HPA-1b will produce anti-HPA-1a antibodies[8,14,27,28].

The discrepancy between the expected and actual incidence of AIT can be explained, at least in part, by the efficiency of antigen presentation by different MHC subtypes. In 1981 Reznikoff-Etievant et al.[29] reported that the immune response to the HPA-1b antigen is associated with the maternal HLA type B8DR3*. Subsequently, several authors confirmed a strong association between the production of anti-HPA-1a antibodies and the HLADR3 locus[28,30–35]. de Waal et al.[36] later linked it to the supertypic HLA DRB3[1]0101 allele.

[1] The original description was of increased incidence of HLA type B8 in women producing anti-HPA-1a antibody. HLA B8 is in linkage disequilibrium with HLA DR3.[25]

The HLA DRB3*0101 locus has a binding affinity for the Leu33 substitution on HPA-1a, and not the Pro33 substitution on HPA-1b, demonstrating the importance of binding between fetal peptides expressed on the β-3 integrin and the maternal MHC locus[37]. This genetic maternal restriction on the immune response to foreign fetal antigens accounts, in large part, for the relatively low number of HPA-1a negative mothers who develop alloantibodies against their HPA-1a positive fetus.

Williamson et al.[14] studied HPA type, antibody production, and maternal HLA type in more than 24 000 consecutive pregnancies in Cambridge and East Anglia, England. Of the 2.5% of women homozygous for HPA-1b, anti-HPA-1a antibodies were detected in 12%. All but one of the antibody producers was HLA DRB3[1]0101 positive, while the overall frequency of the allele was 31.9%. The presence of the HLA DRB3[1]0101 phenotype did not predict alloimmunization (positive predictive value of 35%), but the absence of the HLA DRB3[1]0101 precludes alloimmuniza-

Cambridge Books Online © Cambridge University Press, 2009

tion with very few exceptions (negative predictive value 99.6%).

Although not as well studied, alloantibody formation in cases of HPA-5b incompatibility has been associated with maternal HLA DRw6 positivity[17,18,38]. Other HPA incompatibilities resulting in alloimmunization may have similar HLA associations[39], but the low incidence of AIT due to these antigens makes study difficult.

Allo-antibody mediated thrombocytopenia

Maternal alloimmunization to fetal platelet antigens is most likely necessary, and usually, but not always, sufficient for immune thrombocytopenia in the fetus or newborn. There are a number of reported cases of reasonably high titre maternal anti-platelet alloantibodies in infants with normal platelet counts[14,18,26,27,40]. Maternal alloimmunization without neonatal thrombocytopenia may be as high as 32%[40]. In addition to maternal HLA MHC class II modulation of alloimmunization, there appears to be another regulatory step in AIT. The process of antibody-mediated platelet destruction may be inhibited by unknown factors specific to certain maternal–fetal pairs. It is possible that this factor could be maternal, a so-called 'blocking' antibody.

Conversely, there are many reports of maternal–fetal HPA incompatibility with neonatal thrombocytopenia (presumed AIT), but no detectable maternal alloantibody[9,13,16,40–42]. Newer techniques of antibody detection have not entirely resolved this issue.

For the most part, careful and extensive study of AIT is limited to anti-HPA-1a antibodies. The role of alloantibodies to other fetal platelet antigens is less clear. There are reports of suspected AIT with anti-HLA antibodies[9,43–45], anti-blood group antibodies[9], and anti-platelet-specific glycoprotein antibodies[42]. Of these unconfirmed causes of AIT, anti-HLA antibodies are perhaps the most common. King et al.[45] found no significant relationship between the formation of anti-HLA antibodies and neonatal thrombocytopenia. It is possible that the presence of anti-HLA antibodies in infants with thrombocytopenia is coincidental, as anti-HLA antibodies will be absorbed by HLA antigens in the placenta, or on WBCs, thus minimizing their effect on fetal platelets. At best the role of ant-HLA alloantibodies is unclear, but, when present, they appear to have little effect on neonatal platelet counts[44,45]. It is impossible to exclude that they might have a thrombocytopenic effect in individual cases.

Clearly, the details of the genetic and immunologic controls governing the process of alloimmunization have yet to be completely described. Our ability to predict the occurrence of alloimmunization in cases of feto-maternal platelet antigen incompatibility is limited, as is our ability to predict the presence and severity of thrombocytopenia after alloimmunization occurs.

Clinical presentation

For newborns with unsuspected AIT, evaluation will commonly be initiated by the pediatrician or neonatologist after perinatal identification of petechiae or purpura, or by the incidental finding of thrombocytopenia on a screening complete blood count obtained for a different indication. When defined as either a cord blood platelet count of less than 100, or 150×10^9/l, neonatal thrombocytopenia is reported in 0.5–0.9% of newborns, with severe thrombocytopenia (platelet count less than $50\,000 \times 10^9$/l) in 0.14–0.24%[1,16,27,40,42,46,47] (See Table 38.1). Immune-mediated thrombocytopenia may account for as much as 30% of neonatal thrombocytopenias, occurring in 0.3% of all newborns[40]. A small additional subset of patients will be identified because of a sibling with AIT.

The frequency of hemorrhagic symptoms in AIT is likely overestimated in clinical studies not based on population screening, considering that a substantial proportion of patients with AIT will not have severe thrombocytopenia and may go undetected. For those patients with clinically significant disease, or with neonatal platelet counts less that 100×10^9/l, the incidence of minor hemorrhagic diatheses (petechiae, ecchymoses, or hematomas) is as high as 80%[9,12,16]. The most frequent bleeding symptoms for AIT with clinically significant disease are listed in Tables 38.3 and 38.4.

It is more likely that AIT identified through routine screening will follow a benign course. In the largest study of its kind to date, 65% of infants with persistent in utero exposure to maternal anti-HPA-1a alloantibodies had mild or no thrombocytopenia[14]. While petechiae and ecchymoses are important markers of clinically significant disease, they occur in a minority of patients with AIT. A negative family history of perinatal hemorrhagic symptoms should therefore not prevent the consideration of AIT in subsequent pregnancies. Additionally, 20–40% of thrombocytopenic infants with proven AIT will have other perinatal problems (e.g. poor feeding, low birthweight, cardiac problems, or respiratory problems), which may confuse the etiology of the thrombocytopenia[47].

Neonatal platelet counts can vary from normal to less than 10×10^9/l. When looking at all infants with AIT, as many as 35% may have platelet counts less that 50×10^9/l[14]. While this may represent a small proportion of AIT and an

Cambridge Books Online © Cambridge University Press, 2009

even smaller proportion of live births, when present, and when identified clinically rather than by screening, thrombocytopenia in AIT is usually severe[9,13,14]. In fact, AIT is the most common cause of severe thrombocytopenia in newborns in all studies to date[1,13,16].

Intracranial hemorrhage

The mortality rate with AIT has been reported as high as 15%, but this number does not include clinically unrecognized cases. Almost all deaths are associated with intracranial hemorrhage (ICH)[9,26]. ICH occurs in 10–20% of affected neonates[9,26,48]. In a series of almost 50 000 unselected term neonates, the incidence of ICH was 1 in 1500 live births. Twenty-five per cent occurred in infants with AIT, making it the most common cause of severe ICH in term newborns[49]. Intraventricular hemorrhages are most common, but unifocal, multifocal and large parenchymal hemorrhages have been reported[9,50–53]. The vascular distribution of ICH in alloimmune thrombocytopenia may be expected from the fact that normal hemostasis is maintained, in part, through interaction between platelets and the vascular endothelium. It is unclear if glycoprotein-bound antiplatelet antibodies interfere with the ability of platelets to support the vascular endothelium, but it is likely that decreased platelet numbers significantly compromise the integrity of vessel walls. It is equally possible that minimal trauma can lead to devastating vascular hemorrhage in the developing thrombocytopenic fetus or neonate.

Up to 50% of all reported intracranial hemorrhages in AIT will occur antenatally[9,26,54]. Severe thrombocytopenia may occur at the time of the initial fetal sampling in as many as 50% of affected fetuses, and as early as 16 to 20 weeks gestation[10,21,22,55]. In utero hemorrhages may present at any point in gestation, or even in the newborn period with fetal distress or demise[56,57], fetal or neonatal hydrocephalus[9,57,58], encephalomalacia, intracranial cysts[50,51,59], an abnormal neurological exam, or poor feeding[12,49,54,59,60]. ICH has been reported in association with almost all antigen incompatibilities[61,62], but it is most commonly associated with HPA-1a incompatibility[9,12,54].

In vitro binding of autoantibodies to GPIIb/IIIa may rarely result in an acquired disorder in platelet function[63]. In patients homozygotic for HPA-1a, alloantibodies to HPA-1a will completely eliminate platelet aggregation to all GPIIb/IIIa agonists (except ristocetin), but the effect is much less in heterozygous HPA-1a expression (as in all fetuses with AIT). The pathophysiology mediating this effect is not entirely clear. Glycoprotein IIb/IIIa is the most abundant glycoprotein on the platelet surface, and it is the site of fibrinogen binding. However, antibodies to HPA-1a do not appear to bind near enough to the fibrinogen-binding site to cause a steric hindrance[64]. The vascular distribution of some in utero hemorrhages has led to additional speculation about the possible role of endothelial GPIIb/IIIa in the pathogenesis of intracranial hemorrhages. However, the seemingly equivalent rate of ICH in HPA-3b (GPIIb) incompatibility suggests that this may not be a prominent factor since HPA-3b is not expressed on endothelial cells[65]. In summary, platelet counts less than 20×10^9/l in AIT are permissive but not sufficient for ICH. Anti HPA-1a antibodies do not cause substantial platelet dysfunction and the role of HPA-1a on endothelial cells is uncertain. Other factors that may contribute to the occurrence of ICH are unknown.

Predictors of disease

A goal of many recent screening protocols has been to define prognostic factors that may identify those patients at risk for ICH[14,15,66,67]. As discussed above, the presence of maternal alloantibody does not predict thrombocytopenia. However, recent studies have suggested that high (greater that 1:32) third trimester maternal antibody titre[14,15], and high titres of the IgG3 subclass[67] may predict severe thrombocytopenia. For HPA-5b incompatibilities, neither antibody titre nor subclass appears to predict disease[19].

There is a more than 90% recurrence rate of AIT among siblings. Mildly affected or undiagnosed first-born children may have severely affected siblings. Initial fetal platelet counts of less than 20×10^9/l are reported in 50% of siblings of affected children. Forty-five per cent of second siblings may have platelet counts of less than 20×10^9/l by 24 weeks gestation. However the only significant predictor of severe disease in subsequent affected siblings is antenatal ICH in the previous sibling[55].

Diagnosis

The diagnosis of AIT in a thrombocytopenic infant or fetus requires several laboratory observations. There must be HPA incompatibility between mother and child. Although not always identifiable, even with the most sensitive techniques, a firm diagnosis of AIT requires the identification of maternal antiplatelet alloantibodies. These antibodies should bind to paternal, but not maternal platelets, and they should be specific to the antigen incompatibility in question, including specificity for the glycoprotein on which the incompatible epitope is located.

Cambridge Books Online © Cambridge University Press, 2009

Antigen testing

Only a very few facilities can do both DNA-based and serologic testing for the most frequently encountered HPA antigens. With the development of oligonucleotide probes and the refinement of PCR techniques, it is possible to obtain platelet antigen typing on amniocytes and fetal leukocytes[68–70]. Techniques are also now available for platelet antigen genotyping from dried blood spots on cards to aide in rapid perinatal diagnosis[71].

Recently, several ELISA-based[72,73] and fluorescence-based[74] techniques have been developed for rapid antigen typing on large numbers of samples. ELISA-based kits are also available to screen for common HPA antigens, and to allow accurate testing of the common antigens at smaller centres[73].

Alloantibody testing

Early antibody testing was performed with the platelet suspension immunoflourescent test (PSIFT). The PSIFT technique detects antiplatelet antibodies bound to the surface of platelets, but is not specific for anti HPA alloantibodies[75,76]. In 1987, Kiefel et al.[77] reported accurate alloantibody detection with an enzyme immunoassay, the monoclonal antibody immobilization of platelet glycoprotein assay (MAIPA). This technique, and subsequent refinements, now allows for the identification and quantification of glycoprotein-specific alloantibodies[77–79]. MAIPA can be both utilized during pregnancy to monitor antibody levels, and possibly adapted for large-scale screening programs[80]. Other techniques show promise for large-scale screening programs[5,81].

Who to test

When evaluating the thrombocytopenic newborn, it is important to carefully consider the diagnosis of AIT. We believe that all infants with platelet counts less than 50×10^9/l should be tested for AIT and managed accordingly. Even in patients with mild thrombocytopenia, it is always important to identify those with AIT, because of the implication for subsequent offspring, who may be more severely affected. In general, any thrombocytopenic infant, even with a platelet count greater than 50×10^9/l, without evidence of asphyxia or sepsis, is deserving of evaluation. As many as 30% of infants with AIT will have other neonatal medical problems, which can confuse the diagnosis of AIT. Therefore the diagnosis of other newborn disease, regardless of whether or not they are independent causes of thrombocytopenia, should not prevent an evaluation for AIT, especially with severe thrombocytopenia or ICH. Severe thrombocytopenia and ICH are strongly associated with AIT. In premature infants, where an ICH is likely due to an underdeveloped germinal matrix, AIT should be considered if the ICH is associated with thrombocytopenia, or if there is a parenchymal bleed. Finally, any familial or transient neonatal thrombocytopenia should also be evaluated[47].

Screening programs

Currently there is no consensus on the need for routine, prenatal, maternal platelet antigen phenotyping, or on obtaining routine neonatal platelet counts. AIT is a significant cause of prenatal and perinatal morbidity and mortality, the pathogenesis is relatively well understood, and early intervention can prevent adverse outcomes. Unfortunately, we lack adequate biological markers of severe disease to justify the risk associated with fetal blood sampling and, for the same reasons, it is difficult to justify treating blindly without fetal blood sampling to know that the fetus is or will be severely affected.

The financial and technical burden of screening for all maternal platelet antigens would certainly exceed the resources of any large-scale screening program. Testing would have to be based on the ethnic distribution of the most likely antigen incompatibilities, and the severity of the associated thrombocytopenia. For most populations this would require testing for the HPA-1a antigen. However, such a program would miss incompatibilities among the less common human platelet antigens, some of which can lead to severe disease.

If a screening programme were to identify a woman with the HPA-1b phenotype, what would be the next step to determine the need for treatment? Both anti-HPA-1a antibody and HLA-typing for DRB[1]30101 have been suggested. While high anti-HPA-1a antibody titres may predict severe AIT, low or undetectable titres are not at all predictive of the severity of the thrombocytopenia. In most cases of AIT, alloimmunization is eventually detected but it may require multiple antibody assays throughout the pregnancy and postnatal period. Additionally, there are at least three reported cases (including those in unpublished communications) of alloimmunization to HPA-1a in mothers that do not have the HLA DRB[1]30101 subtype.

Screening programs have the potential to prevent the devastating or fatal hemorrhagic complications of AIT. However, without better markers for severe thrombocytopenia in affected maternal–fetal pairs, we have to rely on fetal blood sampling (FBS) to confirm the diagnosis. Until there is better data on the incidence and significance of the

Cambridge Books Online © Cambridge University Press, 2009

biological markers for alloimmunization and thrombocytopenia, it is difficult to justify the number of unnecessary fetal blood samplings that would result from a screening programme using the current technology. For example, a reasonable screening programme might suggest FBS for any maternal–fetal pair with HPA-1a antigen incompatibility and a detectable maternal antibody. If we assume that there is a human–platelet antigen incompatibility in 1 in 40 pregnancies, and that 2% of these will have detectable alloimmunization[14,26–28] then 1 in 2000 pregnancies will result in alloimmunization, and require FBS. Only 35% of infants exposed to antiplatelet alloantibodies will develop moderate to severe thrombocytopenia[14], while only 10–20% of these neonates with suffer an intracranial hemorrhage[9,12,48]. If the fetal loss rate after FBS is 1–2%[82–84], then a screening program will result in fetal loss from FBS in as many as 1 in 100 000 to 1 in 200 000 pregnancies, while potentially preventing ICH in 1 in 28 500 to 1 in 57 000 pregnancies. In other words, in the setting of routine screening of first pregnancies, the best technology available still requires that one in three infants will undergo unnecessary FBS. Additionally, for every infant that suffers a fatal complication of FBS (assuming only one FBS per patient), we will only have the opportunity to potentially prevent ICH in two to five infants.

There are two large, multi-centred studies nearing completion in Northern Europe to help better define these issues.

Management

Ideally, the goal in the management of AIT is to prevent antenatal or perinatal intracranial hemorrhage, and the associated neurologic sequelae or mortality. For those patients diagnosed in the newborn period, prompt diagnosis and treatment is necessary to restore normal hemostasis. However, often the damage is already done and optimal neonatal management merely serves to limit the extent of damage by preventing further progression of the ICH[54]. A number of cases may be diagnosed antenatally because of a history of AIT in a first or second degree relative, or, infrequently, because of an in utero intracranial hemorrhage diagnosed by prenatal ultrasound. Antenatal management of affected fetuses identified because of previously affected siblings can significantly reduce the morbidity and mortality associated with AIT. While severe thrombocytopenia and ICH occur in a minority of patients with AIT, the sequelae are potentially devastating and warrant aggressive preventative efforts.

Antenatal management

There are several important considerations in developing antenatal management strategies for fetuses suspected of having fetal AIT: the potential severity of the thrombocytopenia; the natural history of fetal AIT; the risk of ICH at a given fetal or neonatal platelet count; the mode of delivery; the morbidity and mortality associated with therapy and therapeutic procedures; and the efficacy of available therapies. The appropriate use of antenatal therapies is best defined in second-affected siblings. In the absence of routine maternal human platelet antigen typing, first-born children are rarely diagnosed before the newborn period.

Severity of disease

Other than antenatal ICH in an older affected sibling, there are no reliable predictors of severe thrombocytopenia in subsequent pregnancies. However, if an older sibling had severe AIT, subsequent infants will have disease that is at least as severe[55,59,61]. To our knowledge there are no reports of untreated fetuses with disease less severe than older affected siblings. As many as 40% of initial fetal platelet counts between 20 and 30 weeks gestation will be lower than the platelet nadir of an older affected sibling[55]. Furthermore, there are no reported cases of ‘spontaneous’ increases in the fetal platelet count, and affected fetuses, who are not initially treated because of adequate fetal platelet counts, will probably have a predictable decrease in their platelet counts. If previous pregnancies resulted only in mild disease, the severity of disease in subsequent pregnancies is impossible to predict.

ICH

‘Spontaneous’ fetal ICH is rare at platelet counts greater than 20×10^9/l. However, the risk of ICH at a given platelet count after vaginal births and after cesarean section has not been studied in randomized controlled trials in AIT. Several early reports of ICH in thrombocytopenic infants after vaginal delivery prompted authors to advocate for scheduled ‘elective’ cesarean section in severely thrombocytopenic infants[85,86]. However, it is still not clear if vaginal delivery is an independent risk factor for ICH in thrombocytopenic neonates[87,88]. While in theory it may seem that cesarean section is safer for thrombocytopenic neonates, this is unproven. Successful antenatal therapy may increase fetal platelet counts to greater than 50×10^9/l, the generally accepted, but unproven, threshold for ‘safe’ vaginal delivery.

Therapeutic procedures

Monitoring the efficacy of therapeutic interventions in fetuses with AIT requires fetal blood sampling using the

Cambridge Books Online © Cambridge University Press, 2009

technique of cordocentesis. There is a reported fetal loss rate of 0.2% to 7.2%, depending on the technique (free-hand technique, fixed-needle technique, or combined technique), operator experience, and underlying fetal disease[67,89–92]. In otherwise healthy but thrombocytopenic fetuses, the risk of fetal loss is likely closer to 1%[82,84]. While some authors report no association between fetal loss and fetal platelet count[67], thrombocytopenic fetuses appear to be at higher risk for exsanguination after FBS[84] and therefore transfusion of maternal or other matched antigen negative platelets is the standard of care.

Efficacy of selected therapies

Intrauterine platelet transfusions

Shortly following the description of the cordocentesis procedure, Daffos et al. described the use of washed maternal platelets injected into the umbilical vein of an infant with AIT[93]. Subsequent reports showed the efficacy of prophylactic HPA-1a negative platelet transfusions in maintaining fetal platelet counts and avoiding hemorrhagic complications[22,94–96]. Unfortunately, transfused platelets have a short half-life and repeat transfusions are required every 5 to 7 days to maintain an adequate platelet count ($>20\times 10^9/\mu l$) in a fetus with severe AIT. Repeated weekly fetal blood samplings for the purpose of platelet transfusions, carry a greatly increased risk of fetal loss or distress.

Intravenous immunoglobulin and corticosteroids

While corticosteroids have shown limited benefit in AIT[83], they do have some efficacy both as primary treatment and as adjunctive treatment to the use of intravenous infusions of gammaglobulin (IVIG). However, the use of maternally administered intravenous immunoglobulin has had the greatest impact on the restoration of adequate fetal platelet counts and the prevention of ICH[97–101]. The mechanism of effect of IVIG is unclear, although IVIG is thought to most likely work by competitively inhibiting the binding of alloantibodies to the $F_c\gamma$ receptor[100,102]. Sufficient doses of IVIG will bind to the $F_c\gamma$ receptors in both the placenta, preventing endocytotic uptake of alloantibodies[103], and fetal mononuclear-phagocytic system, preventing alloantibody mediated platelet destruction[100,102]. The former mechanism, in particular, has indirect support in that the levels of IgG in the maternal circulation are in the range that was shown to competitively inhibit the transport of 90% of anti-D[101,103]. Direct infusion of IVIG into the fetal circulation[104,105] and peritoneum[22] has been reported without great success. Given the efficiency of maternal IVIG transfer across the placenta, and the theoretical benefit of $F_c\gamma$ receptor blockade in the placenta, it is most likely unnecessary to directly infuse IVIG into the fetus. However data on this approach is limited.

Fifteen of the first eighteen maternal–fetal pairs treated with maternally administered IVIG (1gram/kg/week) demonstrated a substantial increase in fetal platelet counts. None of the fetuses or newborns suffered an ICH, even when an antenatal ICH was reported in an older sibling. Almost all of the infants had higher birth platelet counts than did their older siblings. When IVIG was used in combination with 3 to 5 mg/kg/day of dexamethasone, 4 of 5 patients developed oligohydramnios[98,100]. In 1992, Lynch et al.[100] reported that the use of IVIG alone seemed inferior to that of IVIG and steroids. Therefore, a treatment protocol with IVIG plus 1.5 mg/kg/day of dexamethasone was piloted. At this lower dose of dexamethasone, there was no reported oligohydramnios[47,100]. A larger randomized trial then analysed whether there was benefit of this lower dose of steroids in combination with IVIG, but the effect of combined IVIG and dexamethasone 1.5mg/kg was no better than IVIG alone[47].

There are a number of reports of refractory fetal thrombocytopenia despite administration of maternal IVIG[22,100,106–108]. The addition of high dose prednisone (60 mg/day) to weekly IVIG may be effective in patients who do not respond to IVIG alone. This dose of prednisone in combination with IVIG did not cause oligohydramnios[47].

Weekly antenatal IVIG appears to be an effective therapy in preventing in utero and perinatal ICH. However, the variable responses of patients to IVIG necessitates that fetal platelet counts be monitored during the pregnancy by cordocentesis. FBS should be used judiciously for the diagnosis and monitoring of AIT. Operators should be experienced (at least 50 procedures)[47,92,109], and concentrated maternal platelets should be transfused into all fetuses with a platelet count less than $50\times 10^9/l$ prior to the removal of the needle. However in the great majority of centres, a platelet count requires 3–5 minutes to be obtained. Therefore, 3 to 10 millilitres of platelets are usually administered prior to knowledge of the fetal platelet count.

Specific management strategies

For the purpose of defining the most safe and effective management strategies for fetuses with known AIT, it is helpful to consider the following: second affected fetuses from the same parents have comparable or more severe disease than their older sibling; thrombocytopenia worsens as the pregnancy progresses; fetal blood sampling

Cambridge Books Online © Cambridge University Press, 2009

Table 38.5. Risk Stratification and initial therapy

	Unknown risk	Standard risk	High risk	Very high risk	Extremely high risk
Antigen incompatibility, but other factors unknown?	**Yes**	No	No	No	No
Known AIT, platelet count $>20\times10^9$, and no history of sibling* with ICH?	No	**Yes**	No	No	No
Platelet count on FBS is $<20\times10^9$, or a history of sibling with perinatal ICH?	No	No	**Yes**	No	No
Sibling with ICH between 28 and 36 weeks gestation?	No	No	No	**Yes**	No
Sibling with ICH occurring before 28 weeks gestation?	No	No	No	No	**Yes**
First fetal blood sampling	See text	20 weeks gestation**	20 weeks gestation	20 weeks gestation	20 weeks gestation
Treatment***	See text	IVIG 0.5g to 1g/kg/week	IVIG 1g/kg/wk, and consider prednisolone 1mg/kg/day	IVIG 1g/kg/wk, to start at 12 weeks gestation	IVIG 1g/kg/wk, to start at 12 weeks gestation, but consider IVIG 2g/kg/wk in 2 divided doses

Notes:
* Refers to antigen positive sibling with suspected or confirmed AIT.
** 20 weeks gestation represents the earliest recommended time for FBS in AIT. For standard risk patients, sampling at 20–24 weeks is probably sufficient. See text for more details regarding timing of FBS.
*** Treatment refers to therapy administered to the mother after the first fetal blood sampling, unless otherwise specified.

is the only way to document a response to therapy; maintenance of a fetal platelet count above 20×10^9/l is likely necessary to reduce the risk of ICH; and maintenance of a platelet count above 50×10^9/l is likely necessary for a safe vaginal delivery. To translate these considerations into specific management strategies, our current protocol employs the following grading system: extremely high risk, very high risk, high risk, standard risk and unknown risk (see Table 38.5).

Extremely high risk
Patients are at extremely high risk for an adverse outcome if they are the antigen positive sibling of a fetus who suffered an antenatal ICH before 28 weeks gestation. For this group, 2 g/kg/week (in 2 doses) of maternal IVIG starting at 12 weeks gestation may be more effective than 1 g/kg/week with prednisone.

Very high risk
Fetuses are at very high risk for an adverse outcome if they are antigen positive and have a sibling who suffered an antenatal ICH between 28 and 36 weeks gestation. IVIG at 1g/kg/week starting at 12 weeks gestation may be sufficient in preventing ICH in this group. Monitoring of the fetal platelet count is crucial and in our experience, most patients will later require the addition of 1mg/kg/day of prednisone.

High risk
Fetuses with a sibling who suffered a perinatal ICH, *or* fetuses with a platelet count less than 20×10^9/l, are defined as being at high risk for an adverse outcome. Our ongoing study asked for an initial FBS at 20 weeks gestation and randomized treatment in this group between IVIG alone and IVIG combined with 1 mg/kg of prednisone. It appears that IVIG and prednisone is more effective for the fetus, but also more toxic for the mother.

Standard risk
Fetuses affected by AIT who have no history of a sibling ICH, and have a platelet count greater than 20×10^9/l, have a lesser risk of an adverse outcome. Treatment after FBS is randomized between 1 g/kg/week of maternal IVIG and prednisone 0.5 mg/kg/day.

Unknown risk
When there is maternal–fetal antigen incompatibility, with or without alloantibody formation, there is an unknown risk of an adverse outcome. The literature is fairly clear about the need for close observation of siblings of children

Cambridge Books Online © Cambridge University Press, 2009

with severe AIT. However, there are no absolute indications for antenatal intervention in patients referred with a history of a first or second degree relative with AIT, or in HPA-1a negative primaparous women, even with an affected fetus, unless the fetal platelet count is known to be low. Given the low incidence of alloimmunization in HPA-1a negative mothers, the low incidence of severe thrombocytopenia after alloimmunization[14], and, consequently, the low incidence of ICH, we do not recommend FBS for all such cases. The optimal management remains unknown for the primiparous woman. Paternal phenotype should be ascertained to determine the potential fetal genotypes. If the father is heterozygous for the HPA-1a antigen, fetal genotype should be determined by amniocentesis. FBS should be performed, as early as 20 to 24 weeks gestation, if the following conditions are met (in the absence of a history of AIT in a first degree relative): there is a HPA-1a antigen incompatibility between mother and fetus (by amniocentesis, or if the mother is HPA-1b positive and the father is homozygous for HPA-1a); and maternal alloantibody is detected, irrespective of titre. Maternal alloantibody titres should be tested every 1–3 months until positive, or until 1–2 months postpartum. Alloimmunization may occur at delivery, in which case antibody titres will not rise until the postpartum period, will be subsequent siblings at great risk for thrombocytopenia.

Special considerations

Failure of treatment

After initiation of 1 g/kg/week of maternal IVIG, repeat platelet counts that are lower than previous samplings, or that are below 20×10^9/l, represent refractory disease, and deserve intensification of therapy. In an ongoing clinical trial, our early data suggests that intensification of therapy to 2 g/kg/week of maternal IVIG with or without the addition of 1 mg/kg/day of maternal prednisone may be effective in such refractory cases (unpublished data).

Platelet count less than 10×10^9/l

A platelet count of less than 10×10^9/l, especially if obtained on the first FBS, places the fetus at substantial risk for an adverse outcome. The combination of 1 mg/kg/day prednisone and 1g/kg/week IVIG seems to be more effective in these patients than IVIG alone (preliminary data).

Frequency of fetal blood sampling

The natural history of AIT predicts that the platelet count in an untreated, affected infant will drop by 20 to 25×10^9/l/week. Consequently, for fetal platelet counts of $100–150 \times 10^9$/l, it is reasonable to perform the next FBS within 4 to 6 weeks, and in 3–4 weeks for fetal platelet counts less that 100×10^9/l. FBS cannot be consistently, safely performed prior to 20 to 24 weeks gestation. If the patient is at very high or extremely high risk for an adverse outcome, it is likely that the platelet count will be very low early in gestation, and therapy should be initiated at 12 weeks gestation, 8 weeks prior to the first FBS.

Treatment in the newborn

Early attempts to treat AIT consisted primarily of maternal platelet transfusion[110]. Maternal platelet transfusion or transfusion of HPA-1a negative platelets are still used in severe cases. In the early 1980s there were reports of the successful use of IVIG to treat immune thrombocytopenia in the newborn[111–113]. Corticosteroids are a third option, but are most effective when used in combination with IVIG. Random donor platelets are effective only for brief periods due to rapid alloimmune destruction.

For most infants with presumed AIT, IVIG (1g/kg/dose) will be sufficient in restoring a normal platelet count. The addition of methylprednisolone (1 mg IV every 8 hours) to IVIG may be better than IVIG alone. Recovery of the platelet count can usually be seen within 24 to 72 hours[9,55,114]. For visceral bleeding, including ICH, patients should receive HPA-1a negative platelets (maternal or single donor) in combination with IVIG and methylprednisolone. If HPA-1a negative platelets are not immediately available, random donor platelets may be substituted with frequent assessments of platelet recovery. Most large centres have identified HPA-1a negative donors, and can likely have HPA-1a negative platelets available on short notice.

Conclusions

Alloimmune thrombocytopenia is a disease of varied presentation and course that may have a devastating impact on severely affected infants and their families. While early treatment and intervention may prevent many of the potential adverse sequelae, the prescribed therapy itself is not without significant risk. The capacity exists to effectively treat a great majority of affected fetuses, but we, unfortunately, lack sensitive, reliable and non-invasive indicators of disease severity, and response to therapy.

As understanding of the pathogenesis of this disease broadens, more effective and specific intervention will become available. Future efforts in the study of AIT must focus on: mechanisms of immune modulation in affected

Cambridge Books Online © Cambridge University Press, 2009

and unaffected mismatched maternal–fetal pairs; biologic markers that better define the disease and risk groups; and effective, non-toxic, risk-based therapeutic interventions.

References

1 Burrows RF, Kelton JG. Fetal thrombocytopenia and its relation to maternal thrombocytopenia. *N Engl J Med* 1993;329: 1463–1466.

2 von dem Borne AEG, Decary F. Nomenclature of platelet-specific antigens. *Transfusion* 1990; 30: 477–481.

3 Kelton JG, Smith JW, Horsewood P et al. ABH antigens on human platelets: expression on the glycosyl phosphatidylinositol-anchored protein CD109. *J Lab Clin Med* 1998; 132: 142–148.

4 von dem Borne AEG, Decary F. ICSH/ISBT Working Party on Platelet Serology. Nomenclature of platelet-specific antigens. *Vox Sang* 1990; 58: 176–177.

5 Kunicki TJ, Newman PJ. The molecular immunology of human platelet proteins. *Blood* 1992; 1386–1404.

6 Skupski DW, Bussel JB. Alloimmune thrombocytopenia. *Clin Obst Gyn* 1999; 42: 335–348.

7 Newman PJ. Nomenclature of human platelet alloantigens: a problem with the HPA system? *Blood* 1994; 83: 1447–1451.

8 Blanchette VS, Petes MA, Pegg-Feige K. Alloimmune thrombocytopenia. *Curr Stud Hematol Blood Transfus* 1988; 55: 87–96.

9 Mueller-Eckhardt C, Kiefel V, Grubert A et al. 348 cases of suspected neonatal alloimmune thrombocytopenia. *Lancet* 1989; 1: 363–366.

10 Hohlfeld P, Forestier F, Kaplan C, Tissot JD, Daffos F. Fetal thrombocytopenia: a retrospective survey of 5194 fetal blood samplings. *Blood* 1995; 84: 1851–1856.

11 Taaning E, Peterson S, Reinholdt J et al. Neonatal immune thrombocytopenia due to allo- or autoantibodies. *Platelets* 1994; 5: 53–58.

12 Bonacossa IA, Jocelyn LJ. Alloimmune thrombocytopenia of the newborn: neurodevelopmental sequele. *Am J Perinatol* 1996; 13: 211–215.

13 Uhrynowska M, Maslanka K, Zupanska B. Neonatal thrombocytopenia: incidence, serological and clinical observations. *Am J Perinatol* 1997; 14: 4154–4158.

14 Williamson LM, Hackett G, Rennie J et al. The natural history of fetomaternal alloimmunization to the platelet-specific antigen HPA-1a (Pl^{A1}, Zw^{a}) as determined by antenatal screening. *Blood* 1998; 92: 2280–2287.

15 Jægtvik S, Husebekk A, Aune B, Oian P, Dahl LB, Skogen B. Neonatal alloimmune thrombocytopenia due to anti-HPA-1a antibodies; the level of maternal antibodies predicts the severity of thrombocytopenia in the newborn. *Br J Obst Gyn* 2000; 107: 691–694.

16 Uhrynowska M, Niznikowska-Marks M, Zupanska B. Neonatal and maternal thrombocytopenia: incidence and immune background. *Eur J Haematol* 2000; 64: 42–46.

17 Kaplan C, Morel-Kopp C, Kroll H, Kiefel V. Set HPA-5b (Br^{a}) neonatal alloimmune thrombocytopenia: clinical and immunological analysis of 39 cases. *Br J Haematol* 1991; 78: 425–429.

18 Panzer S, Auerbach L, Cechova E et al. Maternal alloimmunization against fetal platelet antigens: A prospective study. *Br J Haematol* 1995; 90: 655–660.

19 Kurz M, Stöckelle B, Eichelberger B, Panzar S. IgG titer, subclass and light-chain phenotype of pregnancy-induced HPA-5b antibodies that cause or do not cause alloimmune thrombocytopenia. *Transfusion* 1999, 39: 379–382.

20 Gruel Y, Biozard B, Daffos F, Forestier F, Caen J, Wautier JL. Determination of platelet antigens and glycoproteins in the human fetus. *Blood* 1986; 68: 488–492.

21 Giovangrandi Y, Daffos F, Kaplan C, Forestier F, Macaleese J, Moirot M. Very early intracranial hemorrhage in alloimmune fetal thrombocytopenia. *Lancet* 1990; 336: 310.

22 Murphy MF, Metcalfe P, Waters AH, Ord J, Hambley H, Nicolaides K. Antenatal management of severe feto-maternal thrombocytopenia: HLA incompatability may affect responses to fetal platelet transfusions. *Blood* 1993; 81: 2174–2179.

23 van Loghem JJ, Dorfmeyer H, van der Hart M, Schreider F. Serological and genetical studies on a platelet antigen (Zw). *Vox Sang* 1959; 4: 161–169.

24 Schulman NR, Marder VJ, Heller MC, Collier EM. Platelet and leukocyte isoantigens and their antibodies: serologic, physiologic, and clinical studies. *Prog Hematol* 1964; 4: 222–304.

25 Flug F, Karpatkin M, Karaptkin S. Should all pregnant women be tested for their platelet PLA (Zw, HPA-1) phenotype? *Br J Haematol* 1994; 86: 1–5.

26 Blanchette VS, Chen L, Freidberg ZS, Hogan VA, Trudel E, Decary F. Alloimmunization to the PL^{A1} platelet antigen: results of a prospective study. *Br J Haematol* 1990; 74: 209–215.

27 Durand-Zaleski I, Schlegel N, Blum-Boisgard C, Uzan S, Dreyfus M, Kaplan C. Screening primiparous women and newborns for fetal/neonatal alloimmune thrombocytopenia: A prospective comparison of effectiveness and costs. *Am J Perinatol* 1996; 13: 423–431.

28 Mueller-Eckhardt C, Mueller-Eckhardt G, Willen-Ohff H, Horz H, Kuenzhen E, O'Neill GJ. Immunogenicity of an immune response to the platelet antigen Zw(a) is strongly associated with HLA-B8 and DR3. *Tissue Antigens* 1985; 26: 71–76.

29 Reznikoff-Etievant MF, Dangu C, Lobet R. HLA-B8 antigen and anti-PlA1 alloimmunization. *Tissue Antigens* 1981; 18: 66–68.

30 Mueller-Eckhardt C. HLA-B8 antigen and anti-PlA1 alloimmunization. *Tissue Antigens* 1982; 19: 154–156.

31 Taaning E, Antosen H, Petersen S, Svejgaard A, Thomsen M. HLA antigens and maternal antibodies in allo-immune neonatal thrombocytopenia. *Tissue Antigens* 1983; 21: 351–359.

32 Decary F. Is HLA-DR3 a risk factor in PLA1-negative pregnant

Cambridge Books Online © Cambridge University Press, 2009

women? *Curr Stud Hematol Blood Transfus* 1986; 52: 78–86.
33 Valentin N, Vergracht A, Bignon JD et al. HLA-DRw52a is involved in alloimmunization against PL^{A1} antigen. *Hum Immunol*, 1990; 27: 73–79.
34 Decary F, L'Abbé D, Tremblay L, Chartrand P. The immune response to the HPA-1a antigen: association with HLA-DRw52a. *Transfus Med* 1991; 1: 55–62.
35 L'Abbé D, Tremblay L, Filion M et al. Alloimmunization to platelet antigen HPA-1a (PlA1) is strongly associated with both HLA-DRB3*0101 and HLA-DQB1*0201. *Hum Immunol* 1992; 34: 107–114.
36 de Waal LP, van Dalen CM, Engelfreit CP, von dem Borne AEG. Alloimmunization against the platelet specific Zw^a antigen, resulting in neonatal alloimmune thrombocytopenia or post-transfusion purpura, is associated with supertypic DRw52 antigen including DR3 and DRw6. *Hum Immunol* 1986; 4: 222–304.
37 Wu S, Maslanka K, Gorski J. An integrin polymorphism that defines reactivity with alloantibodies generates an anchor for MHC class II peptide binding. *J Immunol* 1997; 158: 3221–3226.
38 Mueller-Eckhardt C, Becker T, Weisheit M, Witz C, Santoso S. Neonatal alloimmune thrombocytopenia due to fetomaternal Zw^b incompatibility. *Vox Sang* 1986; 50: 94–96.
39 Westman P, Hashemi-Tavoularis S, Blanchette V et al. Maternal DRB1*1501, DQA1*0102, DQB1*0602 haplotype in fetomaternal alloimmunisation against human platelet alloantigen HPA-6B (GPIIIa-Gln489). *Tissue Antigens* 1997; 50: 113–118.
40 Dreyfus M, Kaplan C, Verdy E, Schlegel N, Durant-Zaleski I, Tchernia G. The Immune Thrombocytopenia Working Group. Frequency of immune thrombocytopenia in newborns: a prospective study. *Blood* 1997; 89: 4402–4406.
41 Mueller-Eckhardt C, Marks H-J, Baur MP, Mueller-Eckhardt G. Immunogenic studies of the platelet-specific Pl^{A1} (Zw^a). *Immunobiology* 1982; 160: 375–381.
42 Sainio S, Järvenpää A, Renlund M, Riikoren S, Terano, Kekomaki R.. Thrombocytopenia in term children: a population based study. *Obst Gyn* 2000; 95: 441–446.
43 Sharon R, Amar A. Maternal anti-HLA antibodies and neonatal thrombocytopenia. *Lancet* 1981; 1313:
44 Marshall LR, Brogden FE, Roper TS, Barr AL. Antenatal platelet antibody testing by flow cytometry – results of a pilot study. *Transfusion* 1994; 34: 961–965.
45 King KE, Kao KJ, Bray PF, Casella JF, Blakemore K, Callan NA, Kennedy SD, Kickler TS. The role of HLA antibodies in neonatal thrombocytopenia: a prospective study. *Tissue Antigens* 1996; 47: 206–211.
46 de Moerloose P, Boehlen F, Exterman P, Hohlfeld P. Neonatal thrombocytopenia: incidence and characterization of maternal antiplatelet antibodies by MAIPA assay. *Br J Hematol* 1998; 100: 735–740.
47 Bussel JB, Berkowitz RL, Lynch L et al. Antenatal management of alloimmune thrombocytopenia: a randomized trial in fifty-five maternal–fetal pairs. *Am J Obst Gyn* 1996; 174: 1414–1423.
48 Pearson HA, Shulman NR, Marder VJ, Cone TE. Isoimmune neonatal thrombocytopenia purpura: clinical and therapeutic considerations. *Blood* 1964; 23: 154–157.
49 Chaoying M, Junwu G, Chituwo. Intraventricular hemorrhage and its prognosis, prevention and treatment in term infants. *J Trop Pediat* 1999; 45: 237–240.
50 Naidu S, Messmore H, Caserta V, Fine M. CNS lesions in neonatal isoimmune thrombocytopenia. *Arch Neurol* 1983; 40: 552–554.
51 Dean LM, McLeary M, Taylor GA. Cerebral hemorrhage in alloimmune thrombocytopenia. *Pediatr Radiol* 1995; 25: 444–445.
52 Johnson JA, Ryan G, al Mussa A,Farkus S, Blanchette US. Fetal diagnosis and management of neonatal alloimmune thrombocytopenia. *Semin Perinatol* 1997; 21: 45–52.
53 Sherer DM Anyaegbunam A, Onyeije C. Antepartum fetal intracranial hemorrhage, predisposing factors and prenatal sonography: a review. *Am J Perinatol* 1998; 7: 431–441.
54 Bussel JB, Tanli S, Peterson HC. Favorable neurological outcome in seven cases of perinatal intracranial hemorrhage due to immune thrombocytopenia. *Am J Pediat Hematol Oncol* 1991; 13: 156–159.
55 Bussel JB, Zabusky MR, Berkowitz RL, McFarland JG. Fetal alloimmune thrombocytopenia. *N Engl J Med* 1997; 337: 22–26.
56 Khouzami AN, Kickler TS, Callan NA, Schuman JB, Perlman EJ, Blakemore KJ. Devastating sequelae of alloimmune thrombocytopenia: an entity that deserves more attention. *J Maternal–Fetal Med* 1996; 5: 137–141.
57 Murphy MF, Hambley H, Nicolaides K, Waters AH. Severe fetomaternal alloimmune thrombocytopenia presenting with fetal hydrocephalus. *Prenatal Diagn* 1996; 16: 1152–1155.
58 Zalneraitis EL, Young RSK, Krishnamoorthy KS. Intracranial hemorrhage in utero as a complication of isoimmune thrombocytopenia. *J Pediat* 1979; 95: 611–614.
59 Herman JH, Jumbelic MI, Ancona RJ, Kickler TS. In utero cerebral hemorrhage in alloimmune thrombocytopenia. *Am J Pediat Hematol Oncol* 1986; 8: 312–317.
60 Morales WJ, Stroup M. Intracranial hemorrhage in utero due to isoimmune neonatal thrombocytopenia. *Obst Gyn* 1985; 65(suppl.): 20–21.
61 Friedman JM, Aster RH. Neonatal alloimmune thrombocytopenic purpura and congenital porencephaly in two siblings associated with a 'new' maternal antiplatelet antibody. 1985; 65: 1412–1415.
62 Jesurun CA, Levin GS, Sullivan WR, Stevens D. Intracranial hemorrhage *in utero* re. Thrombocytopenia [Letter]. *J Pediat* 1980; 97: 695–696.
63 Meyer M, Kirchmaier CM, Schirmer A, Spangenberg P, Strohl C, Breddin K. Acquired disorder of platelet function associated with autoantibodies against membrane glycoprotein IIb–IIIa complex. *Thromb Haemost* 1991; 65: 491–496.
64 Beadling WV, Herman JH, Stuart MJ, Keashen-Schnell M, Miller JL. Fetal bleeding in neonatal alloimmune

Cambridge Books Online © Cambridge University Press, 2009

thrombocytopenia mediated by anti-PLA1 is not associated with inhibition of fibrinogen binding to platelet GPIIb/IIIa. *Am J Clin Path* 1995; 103: 636–641.

65 Glade-Bender G, McFarland J, Kaplan C, Porcelijn L, Bussel J. Anti-HPA-3A induces severe neonatal alloimmune thrombocytopenia: a summary of 30 cases. *J Pediat* (in press).

66 Proulx C, Filion M, Goldman M et al. Analysis of immunoglobulin class, IgG subclass and titre of HPA-1a antibodies in alloimmunized mothers giving birth to babies with or without neonatal alloimmune thrombocytopenia. *Br J Haematol* 1994; 87: 813–817.

67 Weiner MF, Williamson LM, Rodeck CH. Immunoglobulin G subclass of anti-human platelet antigen 1a in maternal sera: relation to the severity of neonatal alloimmune thrombocytopenia. *Eur J Haematol* 1997; 59: 287–292.

68 McFarland JG, Aster RH, Bussel JB, Gianopoulos JG, Derbes RS, Newman RJ. Prenatal diagnosis of neonatal alloimmune thrombocytopenia using allele-specific oligonucleotide probes. *Blood* 1991; 78: 2276–2282.

69 Skogen B, Bellissimo DB, Hessner MJ et al. Rapid determination of platelet alloantigen genotyped by polymerase chain reaction using allele-specific primers. *Transfusion* 1994; 34: 955–960.

70 Avent ND. Antenatal genotyping of the blood groups of the fetus. *Vox Sang* 1998; 74(Suppl. 2): 365–374.

71 Hughes D, Hurd C, Williamson LM. Genotyping for human platelet antigen-1 directly from dried blood spots on cards. *Blood* 1996; 88: 3242–3243.

72 Bessos H, Mirza S, McGill A, Williamson LM, Hatfield R, Murphy WG. A whole blood assay for platelet HPA-1 (PL^{A1}) phenotyping applicable to large scale antenatal screening. *Br J Haematol* 1996; 92: 221–225.

73 Bessos H, Hofner M, Salamat A, Wilson D, Urbaniak S, Turner ML. An international trial demonstrates suitability of a newly developed whole-blood ELISA kit from multicentre platelet HPA-1 phenotyping. *Vox Sang* 1999; 77: 103–106.

74 Quintanat A, Jallu V, Legros Y, Kaplan C. Human platelet antigen genotyping using a fluorescent SSCP technique with an automatic sequencer. *Br J Haematol* 1998; 103: 437–444.

75 von dem Borne AEG, Verheught FWA, Oosterhof F, von Riesz E, Brutel de la Riviere A, Engelfriet CP. A simple immunofluorescence test for the detection of platelet antibodies. *Br J Haematol* 1978; 31: 195–207.

76 von dem Borne AEG, van Leeuwen EF, von Riesz LE, van Boxtel CJ, Engelfriet CP. Neonatal alloimmune thrombocytopenia: detection and characterization of the responsible antibodies by the platelet immunofluorescence test. *Blood* 1981; 57: 649–656.

77 Kiefel V, Santoso S, Weishert M, Mueller-Eckhardt C. Monoclonal antibody-specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet-reactive antibodies. *Blood* 1987; 70: 1722–1726.

78 Morel-Kopp MC, Kaplan C. Modification of the MAIPA technique to detect and identify anti-platelet glycoprotein autoantibodies. *Platelets* 1994; 5: 285.

79 Jousti L, Kekomäki R. Comparison of the direct platelet immunofluorescence test (direct PIFT) with a modified direct monoclonal antibody-specific immobilization of platelet antigens (direct MAIPA) in detection of platelet-associated IgG. *Br J Haematol* 1997; 96: 204–209.

80 Dawkins B. Monitoring anti-HPA-1a platelet antibody levels during pregnancy using the MAIPA test. *Vox Sang* 1995; 68: 27–34.

81 Metcalf P, Doughty HA, Murphy MF, Waters AH. A simplified method for large-scale HPA-1a phenotyping for antenatal screening. *Transfus Med* 1994; 4: 21–25.

82 Pielet BW, Socol ML, MacGregor SN, Ney JA, Dooley SL. Cordocentesis: an appraisal of risks. *Am J Obstet Gynecol* 1988; 159: 1497–1500.

83 Daffos R, Forestier F, Kaplan C. Prenatal treatment of fetal alloimmune thrombocytopenia [Letter]. *Lancet* 1988; 2: 910.

84 Paidas MJ, Berkowitz RL, Lynch L et al. Alloimmune thrombocytopenia: fetal and neonatal losses related to cordocentesis. *Am J Obst Gyn* 1995; 172: 475–479.

85 Sitarz AL, Driscoll JM, Wolff JA. Management of isoimmune neonatal thrombocytopenia. *Am J Obst Gyn* 1976; 124: 39–42.

86 Murray JM, Harris RE. The management of the pregnant patient with idiopathic thrombocytopenic purpura. *Am J Obst Gyn* 1976; 126: 449–451.

87 Sia CG, Amigo NC, Harper RG, Farahani G. Failure of cesarean section to prevent intracranial hemorrhage in siblings with isoimmune thrombocytopenia. *Am J Obst Gyn* 1985; 153: 79–81.

88 Cook RL, Miller R, Katz VL, Cefalo RC. Immune thrombocytopenic purpura in pregnancy: a reappraisal of management. *Obst Gyn* 1991; 78: 578–583.

89 Buscaglia, M Ghisoni L, Bellotti M et al. Percutaneous umbilical blood sampling: indication changes and procedure loss rate in a nine years' experience. *Fetal Diagn Ther* 1996; 11: 106–113.

90 Petrikovsky B, Schneider EP, Klein VR, Wyse LJ. Cordocentesis using the combined technique: needle guide-assisted and free-hand. *Fetal Diagn Ther* 1997; 12: 252–254.

91 Antsaklis A, Daskalakis G, Papantoniou N, Michalas S. Fetal blood sampling–indication-related losses. *Prenatal Diagn* 1998; 18: 934–940.

92 Tongsong T, Wanapirak C, Kunavikatikul C, Sirichotiyakul S, Piyamongkol W, Chanprapaph P. Cordocentesis at 16–24 weeks of gestation: experience of 1320 cases. *Prenat Diagn* 2000; 20: 224–228.

93 Daffos R, Forestier F, Muller JY et al. Prenatal treatment of alloimmune thrombocytopenia. *Lancet* 1984; 2: 632.

94 Kaplan C, Forestier F, Cox WL, Lyon-Caen D, Dupuy-Montburn MC, Salmon C. Management of alloimmune thrombocytopenia: antenatal diagnosis and in utero transfusion of maternal platelets. *Blood* 1988; 72: 340–343.

95 Nicolini U, Rodeck CH, Kochenour NK, Grenco P, Fisk NM, Letsky E. In-utero platelet transfusion for alloimmune thrombocytopenia [Letter]. *Lancet* 1988; 2: 506.

96 Murphy MF, Waters AH, Doughty HA et al. Antenatal manage-

Cambridge Books Online © Cambridge University Press, 2009

ment of fetomaternal alloimmune thrombocytopenia-report of 15 affected pregnancies. *Transfus Med* 1994; 4: 281–292.

97 Suarez CR,l Anderson C. High dose intravenous gammaglobulin (IVIG) in neonatal immune thrombocytopenia. *Am J Hematol* 1987; 26: 247–253.

98 Bussel, JB, Berkowitz RL, McFarland JG, Lynch L, Chitkara U. Antenatal treatment of neonatal alloimmune thrombocytopenia. *N Engl J Med* 1988; 319: 1374–1378.

99 Wenstrom KD, Weiner CP, Williamson RA. Antenatal treatment of fetal alloimmune thrombocytopenia. *Obst Gyn* 1992; 80: 433–435.

100 Lynch L, Bussel JB, McFarland JG, Chitkara U, Berkowitz RL. Antenatal treatment of alloimmune thrombocytopenia. *Obst Gyn* 1992; 80: 67–71

101 Bussel JB, Berkowitz RL, McFarland JG. Maternal IVIG in neonatal alloimmune thrombocytopenia. *Br J Haematol* 1997; 98: 493–494.

102 Debré M, Bonnet MC, Fridman WH et al. Infusion of Fcγ fragments for treatment of children with acute immune thrombocytopenic purpura. *Lancet* 1993; 342: 945–949.

103 Urbaniak SJ, Duncan I, Armstrong-Fisher SS, Abramovich DR, Page KR. Transfer of anti-D antibodies across the isolated perfused human placental lobule and inhibition by high dose intravenous immunoglobulin: a possible mechanism of action. *Br J Haematol* 1997; 96: 186–193.

104 Bowman J, Harman C, Mentigolou S, Pollock J. Intravenous fetal transfusion of immunoglobulin for alloimmune thrombocytopenia. [Letter]. *Lancet* 1992; 340: 1034.

105 Zimmerman R, Huch A. In-utero fetal therapy with immunoglogulin for alloimmune thrombocytopenia [Letter]. *Lancet* 1992; 340: 606.

106 Mir N, Samson D, House MJ, Kovar IZ. Failure of antenatal high-dose immunoglobulin to improve fetal platelet count in neonatal alloimmune thrombocytopenia. *Vox Sang* 1988; 55: 188–189.

107 Nicolini U, Tannirandorn Y, Gonzalez P, Fisk NM, Beacham J, Lesky EA, Rodeck CH. Continuing controversy in alloimmune thrombocytopenia: fetal hyperimmunoglobulin fails to prevent thrombocytopenia. *Am J Obst Gyn* 1990; 163: 1144–1146.

108 Kroll H, Kiefel V, Griers G et al. Maternal intravenous immunoglobulin treatment does not prevent intracranial hemorrhage in fetal alloimmune thrombocytopenia. *Transfus Med* 1994; 4: 293–296.

109 Bussel JB, Skulpski DW, McFarland JG. Fetal alloimmune thrombocytopenia: consensus and controversy. *J Maternal–Fetal Med* 1996; 5: 281–292.

110 Adner MM, Fisch GR, Starobin SG, Aster RH. Use of 'compatible' platelet transfusions in treatment of congenital isoimmune thrombocytopenic purpura. *N Engl J Med* 1969; 280: 244–247.

111 Sidiropoulos D, Straume B. The treatment of neonatal isoimmune thrombocytopenia with intravenous immunoglobin (IgG i.v.). *Blut* 1984; 48: 383–386.

112 Derycke M, Dreyfus M, Ropert JC, Tchermia G. Intravenous immunoglobulin for neonatal isoimmune thrombocytopenia. *Arch Dis Childr* 1985; 60: 667–669.

113 Massey GV, McWilliams NB, Mueller DG, Napolitano A, Maurer HM. Intravenous immunoglobulin in neonatal isoimmune thrombocytopenia. *J Pediat* 1987; 111: 133–135.

114 Bussel JB, Kaplan C, McFarland J. Working Party on Neonatal Immune Thrombocytopenia of the Neonatal Hemostasis Subcommittee of the Scientific and Standardization Committee of the International Society of Thrombosis and Haemostasis: Recommendations for the evaluation and treatment of neonatal autoimmune and alloimmune thrombocytopenia. *Thromb Haemost* 1991; 65: 631–634.

115 Maslanka K, Lucas GF, Gonkowska A, Davis JG, Zupanska B. A second case of neonatal alloimmune thrombocytopenia associated with anti-PL^{A2} (Zw^b) antibodies. *Haematologia* 1989; 22: 109–113.

116 Kuijpers RW, van den Anker JN, Baerts W, von dem Borne AEG. A case of severe neonatal thrombocytopenia with schizencephaly associated with anti-HPA-1b and antiHPA-2a. *Br J Haematol* 1994; 87: 576–579.

117 Mercier P, Chichepostiche C, Reviron D, Dabanian, Bourgue F, Chiarone J, Diallo M, Delord Y. Neonatal thrombocytopenia in HLA-DR, -DQ, -DP-typed mother due to rare anti-HPA-1b (anti-PL^{A2}) (Zw^b) fetomaternal immunization. *Vox Sang* 1994; 67: 45–51.

118 von den Anker J, Huiskes E, Porcelein L, von dem Bourne AE. Anti-HPA-1b really causes neonatal alloimmune thrombocytopenia. *Br J Haematol* 1995; 89: 428.

119 Winters JL, Jennings CD, Desai NS. Neonatal alloimmune thrombocytopenia due to anti-HPA-1b (PL^{A2}) (Zw^b). *Vox Sang* 1998; 74: 256–259.

120 von dem Borne AEG, von Riesz LE, Verheught FWA, ten Cate JAW, Koppe JG, Engelfriet LP. Bak^a, a new platelet-specific antigen involved in neonatal allo-immune thrombocytopenia. *Vox Sang* 1980; 39: 113–120.

121 Kiefel V, Santoso S, Katxman B, Meuller-Eckhardt C. A new platelet-specific alloantigen Br(a): Report of 4 cases with neonatal alloimmune thrombocytopenia. *Vox Sang* 1988; 54: 101–106.

122 Kekomäki R, Jouhikainen T, Ollikainen J, Westman P, Laes M. A new platelet alloantigen Tu^a, on glycoprotein IIIa associated with neonatal alloimmune thrombocytopenia in two families. *Br J Haematol* 1993; 83: 306–310.

123 McFarland JG, Blanchette V, Collins J, Newman PJ, Wang R, Aster RH. Neonatal alloimmune thrombocytopenia due to a new platelet-specific alloantibody. *Blood* 1993; 81: 3318–3323.

Cambridge Books Online © Cambridge University Press, 2009

39

Drug-induced and drug-dependent immune thrombocytopenias

Andreas Greinacher, Petra Eichler and Norbert Lubenow

Institute for Immunology and Transfusion Medicine, Ernst-Moritz-Arndt-University, Greifswald, Germany

Introduction

This chapter will focus on pathological conditions of enhanced platelet destruction mediated by drug-induced and drug-dependent immune mechanisms. Drug-induced thrombotic thrombocytopenic purpura (TTP) will be outlined in Chapter 40.

Drugs may induce various immune-mediated hemocytopenias, immune hemolytic anemia[1], neutropenia[2,3] and thrombocytopenia (Table 39.1).

Table 39.1. Immune thrombocytopenias induced by drugs

1. drug induced autoimmune thrombocytopenia
2. drug dependent immune thrombocytopenia
3. GPIIb/IIIa-antagonist induced thrombocytopenias
4. heparin-induced thrombocytopenia (HIT)
5. drug-induced thrombotic thrombocytopenia (TTP)

Drug-induced autoimmune thrombocytopenia

Von dem Borne et al.[4] reported gold therapy-induced antibodies that bind to platelet membrane targets even when the drug is absent. This has been confirmed by several other groups[5]. The clinical presentation of these patients is indistinguishable from autoimmune thrombocytopenia. Even after cessation of gold therapy, the autoantibodies cause destruction of platelets, usually by phagocytosis in the reticulo-endothelial system (RES). Autoantibody titres may decrease slowly, within months, after cessation of gold therapy paralleled by an increase in platelet counts. Laboratory diagnosis of these antibodies does not differ from other platelet autoantibodies (see Chapter 36). The same is true for treatment. However, gold should be avoided in these patients[6].

Drug-dependent immune thrombocytopenias (ddtp)

Pathogenesis

In ddtp the antibodies only bind to platelets in the presence of the drug or of one of its metabolites. Several models of pathogenesis have been debated[7] (Figure 39.1). According to the hapten-theory[8–10] the antibodies recognize an epitope on the drug. If the drug binds to the platelet surface, the antibodies also bind to the surface bound drug which results in platelet destruction in the RES. This mechanism can be responsible for penicillin-induced thrombocytopenia,[9] but is very unlikely to be the mechanism of other ddtps. Haptens usually bind strongly to proteins and cells[9]; binding of most drugs to platelets, however, is weak. They can be washed off cells rather easily[11,12]. Furthermore, in the test tube, high concentrations of the drug in the supernatant do not block antibody binding[6].

As early as 1963 Shulman and Reid[13] presented an immune-complex mechanism, according to which the drug binds to a protein. The multimolecular complex of protein, drug and antibodies should bind to platelets, causing platelet destruction[14]. Ddtp-antibodies, however, bind to platelets by their Fab-parts and not via their Fc-part[15,16].

According to the trimolecular-complex theory the epitope in ddtp is formed after binding of a drug to a platelet glycoprotein. It still remains unresolved whether the antibodies recognize a change of protein conformation, or the complex of protein and drug. Ddtp-antibodies typically react with monomorphic epitopes on platelet glycoproteins but only in the presence of the drug or a

metabolite. Although several platelet glycoproteins (GPs) have been identified as the antibody target in ddtp (GPIb/IX[17–19], GPV[20], GP IIb/IIIa[21–25]) antibodies in an individual patient are highly specific for a single GP.

Kroll et al.[26] investigated sera of 5 patients with carbimazole-dependent platelet reactive antibodies. They identified a complex of PECAM-1 (platelet endothelial cell adhesion molecule-1, CD 31) and carbimazole to be the antibody binding site.

Drugs causing ddtp

Many drugs in numerous case reports have been suspected to cause ddtp (for a reference list of all case reports until 1998 see: *http: //www.acponline.org/journals/annals/01dec98/drugdata.htm*

http: //www.acponline.org/journals/annals/01dec98/drugindu.htm

George et al.[5,27], but only a few have been documented by appropriate laboratory studies[28]. In some cases a metabolite rather than the drug itself mediates ddtp antibody binding to platelets[12,29–31]. Table 39.2 summarizes all drugs suspected to induce ddtp.

During a time period of 5 years, our laboratory had 153 requests to test for ddtp antiplatelet antibodies. In 11 of them a clear positive result could be obtained: in six patients with carbamazepine, and in one patient each with ceftriaxone, trimethoprim, sulfamethoxazole, perazin, piperacilline/tazobactam. To put these data in perspective, during the same time period, heparin-induced thrombocytopenia antibodies had been confirmed in 1189 patients in our laboratory.

Clinical presentation

Clinical symptoms of ddtp typically occur after an interval of at least 7–10 days after start of medication. As ddtp antibodies may persist for years, in case of re-exposure symptoms can manifest within hours. Platelet counts typically decrease to values below 20 000/μl, petechiae and hemorrhagic blisters of the mucosa are frequent[5,32]. An exception are carbimazole-dependent antibodies[26], which cause a moderate decrease of platelet counts only. The most likely reason is that PECAM-1, which is involved in antigen presentation, also is expressed on leukocytes and endothelial cells. Thus the drug-induced antibodies bind to non-platelet epitopes, too. Whether this mechanism is restricted to carbimazole or applies to other drugs as well is unresolved.

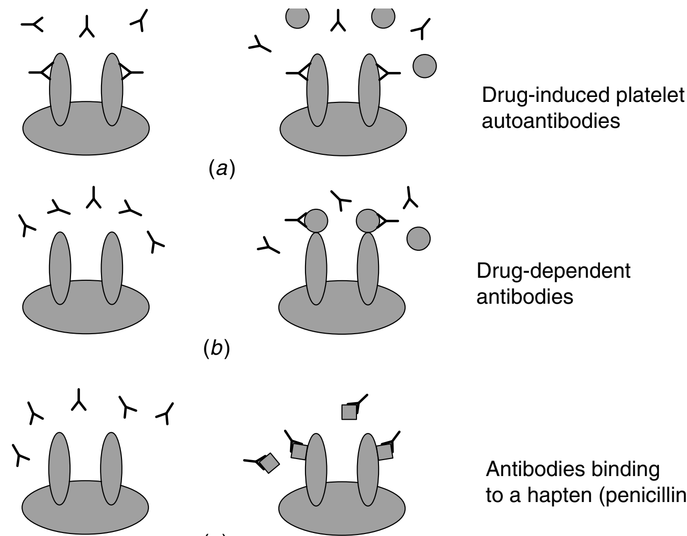


Fig. 39.1. Different hypotheses for drug dependent immune thrombocytopenias (ddtp) are described. (*a*) Drug-induced platelet autoantibodies bind to platelets in the absence (left side) as well as in the presence of the causative drug (right side). (*b*) In the absence of the causative drug, ddtp-antibodies are not able to bind to platelets (left side) but in the presence of the drug the antibodies form a labile trimolecular complex with the drug and platelet membrane structures (right side). (*c*) In the classical hapten reaction, ddtp-antibodies bind to haptens in plasma or to haptens on the cell membrane. In vitro antibody-binding to platelets is inhibited by excess drug.

Treatment

The most important measure in ddtp is cessation and future avoidance of the responsible drug. In the experience of the authors, platelet counts usually rise after 4–5 drug half-life times to values above 50,000/μl. In the meantime, general measures to avoid high intracranial pressure are indicated. If hemorrhage is life-threatening, massive platelet transfusions and i.v. IgG should be considered.

Diagnosis of ddtp

Most important for the diagnosis of ddtp is the clinical judgement and a carefully taken history. Drugs taken by the patient for months or even years are rarely the cause for the immune response. Drugs taken during the last 2 weeks are much more likely to be the trigger. In general drugs taken before, not after, onset of symptoms, are of major relevance.

Cambridge Books Online © Cambridge University Press, 2009

Table 39.2. Number of case reports for specific drugs (in parentheses) according to George et al.[5]

Drug	Drug	Drug
Abciximab c7E3 Fab (7)	Cyclophosphamide (1)	Isoniazid (1)
Acetaminophen (10)	Cyclosporin (2)	Isotretinoin (2)
Acetazolamide (3)	Dactinomycin-actinomycin (3)	Itraconazole (1)
Allopurinol (3)	Danazol (7)	l-tryptophan (1)
Allylisopropyl acetylurea (1)	Deferoxamine (1)	Levadopa (1)
Alprenolol (1)	Desipramine (2)	Levamisole (4)
Aminoglutethimide (5)	Diatrizoate meglumine (4)	Lidocaine (1)
Aminosalicylic acid (5)	Diatrizoate meglumine-	Lincomycin (1)
Amiodarone (2)	diatrizoate sodium (2)	Lithium (2)
Amitriptyline (1)	Diazepam (3)	Lorenzo's oil (2)
Amphotericin B (3)	Diazoxide (2)	Mainserin (1)
Ampicillin (4)	Diclofenac (6)	Mebhydrolin (1)
Amrinone (3)	Didanosine (1)	Meclofenamate (2)
Anagrelide (1)	Diethylstilbestrol (2)	Mefenamic acid (1)
Anorexigenic agents (1)	Difluoromethylornithine (2)	Mercurial diuretics (1)
Antilipemic agents (3)	(Eflornithine)	Mesalamine (1)
Antithyroid agents (1)	Diflunisal (1)	Methazolamide (1)
Apalcillin (1)	Digoxin (6)	Methicillin (1)
Arsphenamine (14)	Diltiazam (4)	Methotrimeprazine (3)
Aspirin (3)	Doxepin (2)	Methyldopa (8)
Benoxaprofen (2)	Enalapril (1)	Methylphenidate (1)
Benzodiazepines (1)	Erucic acid (6)	Mexiletine (2)
Bismuth (1)	Ethambutol (2)	Mezlocillin (2)
Bleomycin (8)	Ethchlorvynol (1)	Minoxidil (1)
Bromocriptine (1)	Ethylenedinitrilo tetraacetic acid (1)	Morphine (1)
Bumetanide (1)	Etretinate (2)	Moxalactam (1)
Butabarbital (1)	Famotidine (4)	Nalidixic acid (6)
Butoconazole (1)	Felbamate (1)	Naphazoline (2)
Captopril (5)	Fenoprofen (2)	Naproxen (2)
Carbamazepine (16)	Feprazone (1)	Nifedipine (1)
Carbimazole (1)	Flavone-8-acetic acid (1)	Nimesulide (1)
Carboplatin (1)	Fluconazole (3)	Nitroglycerine (1)
Cefamandole (1)	Flucytosine (1)	Nomifensine (1)
Cefotetan (2)	Fluphenazine (1)	Nonsteroidal
Ceftazidime (1)	Furosemide (1)	antiinflammatory drugs (1)
Ceftriaxone (1)	Fusidic acid (2)	Novobiocin (1)
Cefuroxime (1)	Ganciclovir (1)	Octreotide (1)
Cephalothin (3)	Gentamicin (1)	Ondansetron (6)
Chlorothiazide (15)	Glibenclamide (3)	Oxyprenolol (3)
Chlorpheniramine (1)	Gold (80)	Oxytetracycline (3)
Chlorpromazine (8)	Haloperidol (3)	Oxyphenbutazone (3)
Chlorpromazine (8)	Hydralazine (1)	Papaverine (1)
Cimetidine (23)	Hydrochlorothiazide (6)	Penicillamine (3)
Ciprofloxacin (1)	Hydroxychloroquine (1)	Penicillin (3)
Clarithromycin (1)	Hydroxyurea (1)	Pentagastrin (1)
Clidinium bromide-	Ibuprofen (3)	Pentamidine (1)
chlordiazepoxide (1)	Indomethacin (3)	Phenobarbital (1)
Clonazepam (1)	Interferon-α (8)	Phenybutazone (1)
Clozapine (2)	Iocetamic acid (2)	Phenytoin (25)
Cocaine(12)	Iopanoic acid (3)	Phthalazinol (1)
Cyanidanol (1)	Iron dextran (1)	Piperacillin (2)

Cambridge Books Online © Cambridge University Press, 2009

Table 39.2. (*cont.*)

Drug	Drug	Drug
Piperazine (1)	Ranitidine (9)	Suramin (6)
Piroxicam (2)	Remoxipride (1)	Tamoxifen (1)
Plicamycin (1)	Rifampin (17)	Teicoplanin (1)
Podophyllum (1)	Simvastatin (1)	Tetanus oxoid (1)
Potassium iodide (1)	Sodium nitroprusside (2)	Thiotepa (1)
Prednisone (8)	Sodium salicylate (1)	Thiothixene (1)
Primidone (1)	Spiramycin (1)	Ticlopidine (2)
Procainamide (12)	Stibogluconate (2)	Tobramycin (1)
Prochloperazine (1)	Stibophen (2)	Tolbutamide (1)
Propylthiouracil (1)	Sulfadiazine (1)	Tolmetin (2)
Protamine sulfate (1)	Sulfamethoxazole (2)	Trimethoprim sulfamethoxazole (24)
Pyrycinamide (1)	Sulfanilamide (2)	Valproate (3)
Pyrimethamine (2)	Sulfasalazine (10)	Vancomycin (7)
Quiniband (1)	Sulfathiozole (1)	Zidovudine (1)
Quinidine (70)	Sulfisoxazole (2)	
Quinine (24)	Sulindac (3)	

Laboratory diagnosis

Ddtp-antibodies can be confirmed by a variety of assays[33]. Whole platelet-[34] and GP-specific ELISAs[35] have been employed as well as flow cytometry[36], or immunoblot[37]. Due to labile binding of the drug to platelet GPs and the potential dependency of the antigen on protein conformation, assays not altering the GP structure, e.g. flow cytometry are usually more sensitive than immunoblot. In all assays it is absolutely crucial to add to all reaction media, including all washing buffers, with the same concentration of the suspected drug or the metabolite. Appropriate controls are also mandatory (Table 39.3). In case a metabolite of a drug is suspected to be causative, a practical source for some metabolites is the use of urine (pH buffered) obtained from an individual who takes the suspected drug. The clinical importance of negative in vitro tests for drug-dependent antibodies remains uncertain. In some reports, patients with apparent quinidine-induced thrombocytopenia had no demonstrable drug-dependent antibodies using different assays. Therefore, in the absence of promptly available and clinically validated laboratory assays, judgement about ddtp must continue to be based on clinical criteria[38].

GP IIb/IIIa receptor antagonist-induced thrombocytopenias

Frequency

GP IIb/IIIa receptor antagonists are a heterogeneous group of anti-integrilins[39] (Table 39.4) gaining increasing importance in the treatment of acute cardiac syndromes. Thrombocytopenias have been observed in all studies with these drugs, but also occurred in the control populations (Table 39.5). Currently abciximab, tirofiban and oral GPIIb/IIIa antagonists seem to be associated with an increased risk for severe thrombocytopenias, whereas in eptifibatide-treated patients the risk for severe thrombocytopenias was not enhanced as compared to the control population (Table 39.5). In abciximab treated patients severe thrombocytopenias ($<$20 000/μl) occurred in 0.3–1.6 % of patients who received the drug for the first time. If the EPIC, EPILOG and CAPTURE studies are pooled the incidence of severe thrombocytopenias was higher in abciximab treated patients as compared to placebo treated patients (1.1% vs. 0.5%, $P = 0.017$)[49–51].

Clinical presentation and treatment

GP IIb/IIIa inhibitor induced thrombocytopenias typically occur within the first 24 hours after start of treatment. Platelet counts decrease to values below 50 000/μl and

Cambridge Books Online © Cambridge University Press, 2009

Table 39.3. Control experiments for detection of drug-dependent platelet reactive antibodies[32]

Platelets	Drug or metabolite	Serum	Reaction	Interpretation
+	+	Patient	+	Drug-dependent antibody
+	−	Patient	−*	
+	+	Normal donor serum	−*	
+	−	Normal donor serum	−*	
+	+	HPA-antibody-positive serum	+*	
+	−	HPA antibody-positive serum	+*	
+	+	Patient	+	Auto-/alloantibody
+	−	Patient	**+**	
+	+	Normal donor serum	−	
+	−	Normal donor serum−		
+	+	HPA antibody-positive serum	+	
+	−	HPA antibody-positive serum	+	
+	+	Patient	+	Non-specific reaction of the drug with the detection system
+	−	Patient	−	
+	+	Normal donor serum	**+**	
+	−	Normal donor serum	−	
+	+	HPA antibody-positive serum	+	
+	−	HPA antibody-positive serum	+	
+	+	Patient	−	Inhibition of conjugate activity
+	−	Patient	−	
+	+	Normal donor serum	−	
+	−	Normal donor serum	−	
+	+	HPA antibody-positive serum	**−**	
+	−	HPA antibody-positive serum	−	
+	+	Patient	−	Negative result
+	−	Patient	−	
+	+	Normal donor serum	−	
+	−	Normal donor serum	−	
+	+	HPA antibody-positive serum	+	
+	−	HPA antibody-positive serum	+	

Notes:
* control experiments; HPA: human platelet antigen.
+/−: reactions particularly important for interpretation.

Table 39.4. Types of GP IIb/IIIa antagonists

Name	Class	Way of application
Abciximab	Chimeric fab fragment	i.v.
YM337	Humanized fab fragment	i.v
Eptifibatide	Cyclic KGD-peptide	i.v.
Tirofiban	RGD peptido mimetic	i.v.
Lamifiban	RGD peptido mimetic	i.v.
Fradafiban	RGD peptido mimetic	i.v.
Xemilofiban	Peptidomimetic prodrug	oral
Lefradifiban	Peptidomimetic prodrug	oral
Sibrafiban	Peptidomimetic prodrug	oral
DMP 728	Peptidomimetic prodrug	oral

often below 20 000/µl[52]. Patients are at an increased risk to develop bleeding complications. Therefore it is recommended that platelet counts be monitored during treatment with GP IIb/IIIa receptor antagonists. For abciximab, platelet counts are recommend by the manufacturers after 2, 4 and 24 hours.

In our own series of 34 of these patients, hematomas, petechiae, mucosal and urine tract hemorrhages were the most frequent bleeding symptoms. After cessation of the drug, platelet counts normalized rapidly, usually within 2–3 days. Treatment of thrombocytopenia is symptomatic, but the heparin dose should be reduced. In case of bleeding complications, platelet transfusions are the treatment of choice. Steroids or i.v. IgG are not sufficient[53,54]. For

Cambridge Books Online © Cambridge University Press, 2009

Table 39.5. GP IIb/IIIa receptor antagonist induced thrombocytopenias

Study	Study medication	Number of patients	Thrombocytopenia <100 000/μl or >25% (% of patients)	Thrombocytopenia <50 000/μl (% of patients)	Reference
EPIC	Abciximab bolus + infusion	708	5.2	1.6	EPIC Investigators, 1994[40]
	Abciximab bolus	695	3.6	0.3	
	Placebo	696	3.4	0.7	
EPILOG	Abciximab bolus + standard-dose heparin	918	2.6	0.9	EPILOG Investigators, 1997[41]
	Abciximab bolus + low-dose heparin	935	2.5	0.4	
	Placebo + standard-dose heparin	939	1.5	0.4	
CAPTURE	Abciximab bolus + infusion	635	5.6	1.6	CAPTURE Investigators, 1997[42]
	Placebo	630	1.3	0	
IMPACT	Eptifibatide	101	6	0	IMPACT Investigators, 1995[43]
	Placebo	49	0	0	
IMPACT-AMI	Eptifibatide	125	8.8	0	IMPACT-AMI Investigators, 1997[44]
	Placebo	55	14.5	0	
PURSUIT	Eptifibatide	4722	6.8	0.2	PURSUIT Investigators, 1998[45]
	Placebo	4739	6.7	0.1	
RESTORE	Tirofiban	1071	1.1	0.2	RESTORE Investigators, 1997[46]
	Placebo	1070	0.9	0.1	
PRISM	Tirofiban	1616	1.1	0.4	PRISM Investigators, 1998[47]
	Heparin	1616	0.4	0.1	
PRISM PLUS	Tirofiban	345			PRISM-PLUS Investigators, 1998[48]
	Heparin	797	0.8	0.3	
	Tirofiban + heparin	773	1.9	0.5	

platelet transfusion therapy, the different half-lives of the GP IIb/IIIa antagonists and their different pharmacology should be considered. The direct antagonists like abciximab have a high binding affinity to GP IIb/IIIa. Therefore the drug is rapidly cleared from the circulation and redistribution to transfused platelets occurs slowly[55,56]. Eptifibatide and tirofiban, however, have a much lower affinity for the receptor and are given in excess. The GP IIb/IIIa complexes of transfused platelets will be rapidly covered with the drug. Platelet transfusions should therefore be given after several half-lives of the drug have passed following cessation of the drug (4–6 hours). Before heparin dosage is reduced or platelet transfusions are given prophylactically, pseudothrombocytopenia must be excluded.

Pseudothrombocytopenia

This artefact seems to be frequent in abciximab treated patients. Several cases of abciximab induced pseudothrombocytopenia have been described[57–59]. Sane et al.[60] determined incidences of pseudothrombocytopenia and thrombocytopenia in the EPIC-, EPILOG-, EPISTENT- and CAPTURE studies. Pseudothrombocytopenia occurred in 2.1% of abciximab treated patients as compared to 0.6% in placebo treated patients. In our laboratory, in 5 of 34 patients (14.7%) with the clinical suspicion of GP IIb/IIIa antagonist-induced thrombocytopenia, pseudothrombocytopenia was the underlying reason. All these patients had been treated with abciximab.

In our view, pseudothrombocytopenia is the most dangerous side effect of GP IIb/IIIa antagonists. In case of misinterpretation as real thrombocytopenia not only the drug itself but also heparin are often withdrawn and sometimes even platelet transfusions are given. These measures increase the risk of developing a new arterial occlusion. As pseudothrombocytopenia may occur independently of the anticoagulant used, i.e. in EDTA-, citrate- and heparinized blood, a microscopical assessment of a blood smear should always be included in the diagnostic procedure to distinguish between the typical large platelet aggregates and genuine thrombocytopenia. The impressively huge platelet aggregates as seen on blood smears in patients

Cambridge Books Online © Cambridge University Press, 2009

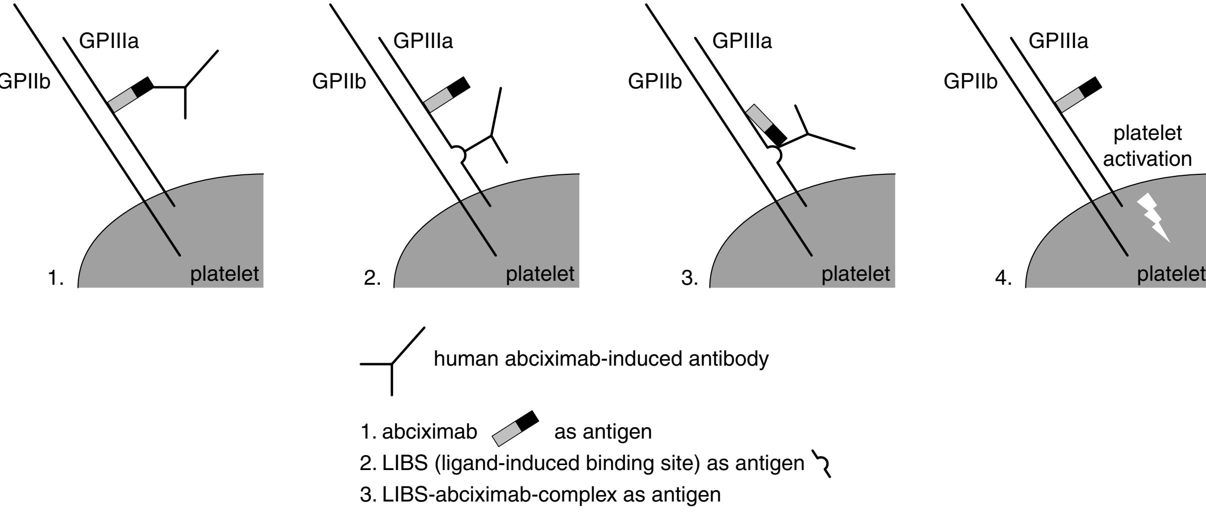


Fig. 39.2. Hypotheses for development of GP IIb/IIIa-induced thrombocytopenia.

with pseudothrombocytopenia, and the still unexplained fact that the large trials of oral GPIIb/IIIa antagonists had to be terminated because of an excess of thrombotic events may be causally related. The exact mechanism of pseudothrombocytopenia in GPII/IIIa treated patients is unknown; however, there is no evidence that other patients with pseudothrombocytopenia, who are not treated with GPIIb/IIIa antagonists, have any clinical sequelae associated with this in vitro phenomenon.

In addition, owing to the concomitant use of heparin and GP IIb/IIIa receptor antagonists, heparin-induced thrombocytopenia should also be considered, if the patient received heparin within the last 3 months before the acute episode.

Pathogenesis

The pathogenesis of GP IIb/IIIa-induced thrombocytopenias is still unresolved. The hypothesis most often presented is that a neoepitope is generated on the platelet surface after binding of the drug. This neoepitope is recognized by preformed antibodies in the patient serum. After binding of these antibodies, platelets are phagocytosed by the RES. Hereby, (i) the drug itself may be recognized as the antigen (Fig. 39.2), (ii) the GP can undergo a conformational change after binding of the drug resulting in a new ligand induced binding site (LIBS)[7,38,61], or (iii) a complex of the drug and the LIBS could be the antigen[38,62].

An alternative theory is that binding of the drug may induce an outside–in signalling which causes platelet activation. The activated platelets would then be destroyed in the RES[63,64].

It seems to be very unlikely that a specific immune response induced by the GP IIb/IIIa antagonists is the reason for the acute thrombocytopenias, as the time interval between start of treatment and onset of thrombocytopenia is too short.

Cines[65] speculated that only in patients with a certain polymorphism of the GP IIb/IIIa complex is a neoepitope expressed after binding of the drug to which preformed antibodies bind by crossreactivity. This model could provide an explanation why only a minority of GP IIb/IIIa receptor antagonist-treated patients develops thrombocytopenia. To test this hypothesis, we screened DNA of 34 affected patients for polymorphisms in the GP IIb/IIIa complex but could not find mutations associated with the occurrence of thrombocytopenia (unpublished).

Curtis et al.[66] used flow cytometry and demonstrated antibodies which bind to abciximab preincubated platelets in 94% of patients with acute thrombocytopenia during abciximab therapy. However, in sera of 10% of normal controls, these antibodies were also present. Whereas the antibodies in the control sera could be neutralized by preincubation with normal Fab fragments, antibodies in patient sera could not. The titres of these antibodies increased tenfold after re-exposure with abciximab.

Kecskes et al.[67] and Cines[65] also described preformed antibodies directed against a conformation-dependent epitope on the GP IIb/IIIa complex. Kroll et al.[68] found

Cambridge Books Online © Cambridge University Press, 2009

abciximab dependent antibodies in 7/7 patients using flow cytometry and the MAIPA technique. However, they found these antibodies also in 17/100 sera of normal blood donors, not pre-exposed to abciximab. We find an even higher percentage of positive results in normal controls. The monoclonal antibody anti-CD41, clone P2 (recognizing GP IIb in the intact GPIIb/IIIa complex) blocks human antibody binding, whereas the moab anti CD-61, clone PM 6/13 (specific for GP IIIa) does not. The epitope involved therefore seems related to the binding site of moab anti-CD41, clone P2. The high percentage of positive results in normal controls, however, raises the question about the diagnostic and pathogenetic relevance of those antibodies.

Kozak et al.[59] demonstrated in vitro platelet aggregation in a patient with abciximab related pseudothrombocytopenia after addition of abciximab, eptifibatide or tirofiban, which they interpreted as crossreactivity of the antibodies.

In our laboratory, antibodies which bound to platelets after preincubation with abciximab did not bind after preincubation with tirofiban. Whether this excludes cross-reactivity, or is a result of the different affinities of the two drugs, is unresolved.

Human antichimeric antibodies (HACAs)

HACAs are induced by the chimeric, humanized Fab fragments of abciximab. Between 4.7 and 6.5% of all patients treated in the EPIC, EPILOG, and CAPTURE studies developed these antibodies, which are directed against the human part of the Fab-fragment. As to be expected from primary immune response, these antibodies occur 2–4 weeks after treatment with a maximal titre after 4–6 weeks[69]. The percentage of patients with HACAs increases after re-exposure to abciximab[70]. However, these antibodies seem to be of very minor, if any, clinical relevance and are not correlated to the risk of occurrence of thrombocytopenia.

Heparin-induced thrombocytopenia

The first case reports of ‘paradox’ thromboembolic complications (TECs) during heparin medication were published in 1958[71]. Today, heparin-induced thrombocytopenia (HIT) is the most important and most frequent drug-induced, immune-mediated thrombocytopenia. This immune HIT has to be distinguished from non-immune interactions between heparin and platelets. In our laboratory confirmed HIT was 100 times more frequent than all other drug-induced thrombocytopenia during the last 5 years.

Non-immune heparin-associated thrombocytopenia

Due to its negative charge, heparin binds to platelets in a saturable manner[72–75]. No unique platelet structures have been identified for this binding and different polysaccharides can displace each other from platelet binding directly dependent on their charge density and chain length[73,76,77]. Numerous in vitro experiments addressed the effects of heparin on platelets[75]. Although results are not always consistent, it is generally agreed that in vitro heparin has a pro-aggregatory effect on platelets[78–80], which is correlated to its grade of sulfation and chain length[81].

These direct heparin–platelet interactions are thought to cause the frequent phenomenon of early onset thrombocytopenia in patients receiving unfractionated heparin (UFH), typically in therapeutic dosage i.v. In a consensus paper[82] it had been suggested to use the term heparin-associated thrombocytopenia to distinguish this syndrome from immune mediated HIT.

Clinically non-immune heparin–platelet interactions cause a mild and transient decrease of platelet counts, rarely $<100\,000/\mu l$[83,84]. In case of certain underlying diseases like anorexia nervosa, burns, arterial disease, or concomitant DIC, platelet counts can decrease to much lower levels[81,85,86].

Diagnosis of non-immune heparin-associated thrombocytopenia is an exclusion diagnosis. Special treatment is usually not required. In patients with a dramatic reduction in platelet counts, low molecular weight heparins (LMWH) may be a therapeutic option for anticoagulation.

These heparin–platelet interactions are of relevance for the development of immune HIT[87]. Binding of platelet factor 4 (PF4) heparin complexes is mediated by the heparin binding site rather than a PF4 binding site[77,88]. Smaller heparin molecules bind less strongly to platelets, cause less platelet activation, less release of PF4 either from platelet alpha granules or from endothelial cell heparan sulfate, and form smaller (less immunogenic) complexes with PF4[89,90].

Immune heparin-induced thrombocytopenia

Pathogenesis of HIT

The pathogenesis of HIT is rather unique. Two compounds which are normally present in the human body, heparin and PF4, complex with each other and induce an immune response.

The proteins involved

Amiral et al.[91,92] were the first who identified PF4 as the most important protein involved in the immune response

Cambridge Books Online © Cambridge University Press, 2009

of HIT and who characterized other involved chemokines as interleukin 8 (IL8) or neutrophil activating peptide-2 (NAP-2)[93] Their finding has been confirmed by several other groups[94–97]. PF4 is a positively charged tetrameric glycoprotein formed by two self-assembling dimers, each consisting of two non-covalently bound monomers. These PF4 tetramers bind to glycosaminoglycans. The amino-acid sequence of PF4 and the encoding DNA sequences are resolved[98,99]. In patients with HIT no mutations of the PF4 gene could be identified[100], thus the antigen is considered to be a cryptantigen or autoantigen exposed after binding of heparin to the tetramer[101]. Horsewood et al.[102] used reduced or alkylated PF4 to map the binding region of HIT antibodies. Of 29 sera, 5 contained antibodies which bound to these modified PF4 proteins and which recognized a 19 COOH-terminal amino acid peptide. By using chimeric PF4[103], PF4 mutants,[104–106] and inhibition experiments[104], the PF4 region between the third and the fourth cysteine residues has been identified as most important for formation of at least three recognition sites for HIT-antibodies. However, as peptides of these regions alone are not able to bind HIT-antibodies, the antigenic site seems to be dependent on the three-dimensional structure of the complex.

The polysaccharides involved

In platelets, eight PF4 tetramers are bound to a chondroitin sulfate proteoglycan[107]. PF4 is released from platelets during platelet activation and can then bind to heparan sulfate containing proteoglycans at the surface of endothelial cells[108]. Polysaccharides with higher sulfation than heparan, e.g. heparin, can displace PF4 from endothelial bound heparan sulfate[109]. The interaction between heparin or other polysaccharides and PF4 has been extensively studied[110]. In essence, the interaction is not dependent on the primary sugar sequence, the type of glycosidic linkage, or the antithrombin binding capacity of a polysaccharide[98,111–113], but on the degree of sulfation and the molecular weight. If the degree of sulfation (number of sulfate groups per monosaccharide) is below 0.5, or the chain length is below 2.4 kDa (~7 monosaccharides) formation of the HIT antibody recognition site after complexing of the polysaccharide with PF4 is very unlikely[111]. Thus the pentasaccharide (1727 kDa)[114], a mixture of low sulfated glycosaminoglycans (danaparoid)[115], or certain synthetic polysaccharides[116] will potentially reduce the risk of immune-mediated HIT.

The concentrations required

Because complexes of PF4 and heparin bind to platelets via the heparin binding site, they have to compete with free heparin[88,117]. If free heparin is in excess, the pathogenic complexes are displaced from the platelet surface. Therapeutic concentrations of heparin reach 0.2–0.4 U/ml, which corresponds to 100–200 nmol/l, whereas the normal PF4 concentration reached after application of heparin is only 8 nM[118] which corresponds to ~0.2 μg/ml. Under in vitro conditions, using 0.2–0.4 IU heparin/ml, the amount of PF4 required to reach the stoichiometric concentrations for the antigenic complex is about 6–20 μg/ml. Thus the HIT antigen is only formed under extreme conditions in which very high amounts of PF4 are released. The situation is very different if small amounts of heparin are given in clinical settings with enhanced platelet activation and higher than normal PF4 concentrations, e.g. during thrombosis prophylaxis after major hip-joint replacement surgery, or in patients with major arterial vessel disease. This seems to be exactly the situation where patients are at the highest risk to develop HIT (Fig. 39.3). Furthermore, this concentration dependency may give an explanation for the differences in the frequency of HIT in different patient populations[75,101].

The antibodies involved

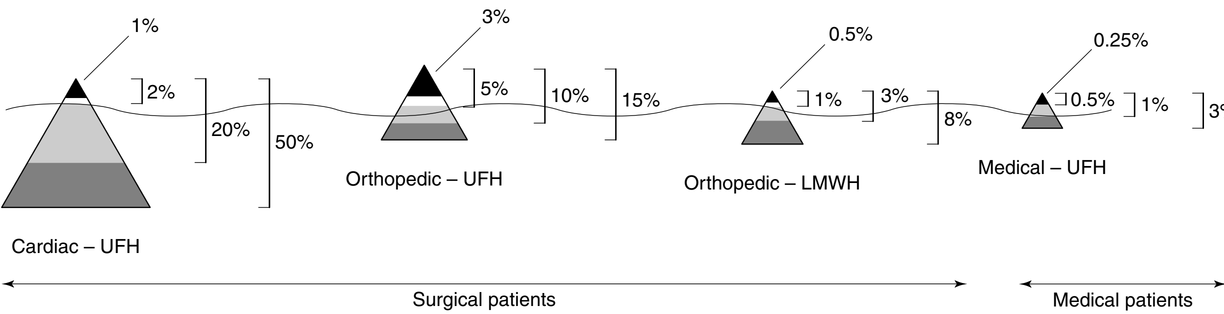
In symptomatic HIT patients, the antibodies involved are of the IgG class in 80% of cases. Antibodies of the IgG1 class were found to be predominant among HIT-IgG[120–122] but patients with HIT antibodies of the IgM or IgA class only have also been described[123]. There is increasing evidence that the percentage of patients developing the immune response of HIT, i.e. a positive HIT-antibody test but no clinical symptoms, is much higher than the percentage of patients who develop clinical HIT. Antibody concentration, antibody affinity, and most importantly the clinical conditions of the patient seem to be relevant for a 'clinical breakthrough' of HIT[101]. Empirically, we have the impression that anti-PF4/heparin antibodies, which activate platelets in an in vitro assay, are more likely to cause clinical HIT than those only detectable by ELISA.

There are pre-existing antibodies against chemokines (IL-8, NAP-2, PF4)[124–126]. Whether they have any pathogenic role in HIT is unresolved.

Platelet receptors and HIT

The most important platelet glycoprotein involved in HIT is the platelet FcγIIa receptor[127]. Binding of HIT antibodies to the PF4–heparin complexes results in large multimolecular immune complexes[94,96]. The Fc-parts of the IgG in the immune complexes crosslink the FcγRIIa. The resulting platelet activation can be completely blocked by a monoclonal antibody against the FcγRIIa[79,128]. The number of FcγRIIa copies on the platelet surface is increased in

Cambridge Books Online © Cambridge University Press, 2009



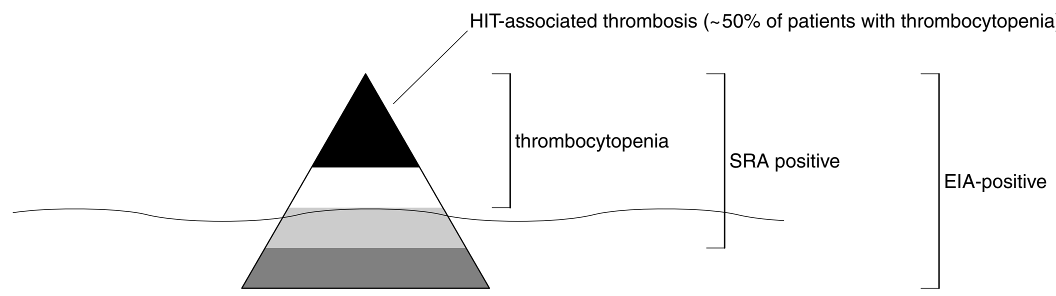


Fig. 39.3. Iceberg-model of heparin-induced thrombocytopenia[119]. SRA: serotonin release assay; EIA: enzyme linked immune assay.

HIT[129], most likely as a marker of increased platelet activation. Soluble FcγRIIa, produced by alternative splicing[130,131], reach about 10% of the total number of the platelet membrane bound receptors. They are not able to saturate the Fc-parts of the immune complexes. As a marker of increased platelet activation, soluble FcγRIIa is found in increased concentrations in HIT patients[132].

The FcγRIIa has a functionally relevant polymorphism, FcγRIIa–Arg/His131, which results in different affinities for human IgG2[133–135]. In vitro, washed platelets with the FcγRIIa His131 variant are preferentially activated by HIT antibodies[121,136]. Several studies investigated the role of this polymorphism in HIT. Burgess et al.[137] ($n=19$), Brandt et al.[138] ($n=96$), Denomme et al.[121] ($n=84$) found the His131 variant overrepresented whereas Arepally et al.[120] ($n=36$) and Bachelot-Loza et al.[136] ($n=25$) found no association between Fcγ RIIa genotype and HIT. Carlsson et al.[139] ($n=389$) showed an increase in the frequency of Arg131. This increase occurred only in the subset of patients with HIT and thrombosis. They hypothesize that the PF4–heparin–HIT–IgG immune complexes and platelets covered with these immune complexes are cleared by the FcγRIIa of the RES more efficiently in individuals with the His131 genotype. Therefore, platelet activating immune complexes circulate longer in patients with the Arg 131 phenotype, thereby enhancing thrombin generation and increasing the risk for HIT-related thrombosis.

Apart from the FcγRIIa, at least one of the platelet ADP receptors seems to be of major importance in HIT, as blocking of the receptor completely inhibits FcγRIIa-mediated platelet activation[140].

Endothelial cells in HIT

HIT antibodies of the IgG, IgA, and IgM class bind to endothelial cells[141], dependent on endothelial cell heparan sulfate and on addition of PF4[96,141]. Activation of endothelial cells by HIT antibodies, however, seems to require cofactors released by activated platelets and can be inhibited by addition of apyrase or a GPIIb/IIIa inhibitor[142]. Although endothelial cells should have an important role in HIT, the exact mechanism is still unresolved.

Thrombin in HIT

Thrombin plays a central role in HIT-related thromboembolic complications (TECs) (Fig. 39.4). Thrombin generation is enhanced in HIT[143,144] by concomitant activation of platelets[145], generation of platelet microparticles[146], and alteration of endothelial cells[141]. Activated endothelial cells

Cambridge Books Online © Cambridge University Press, 2009

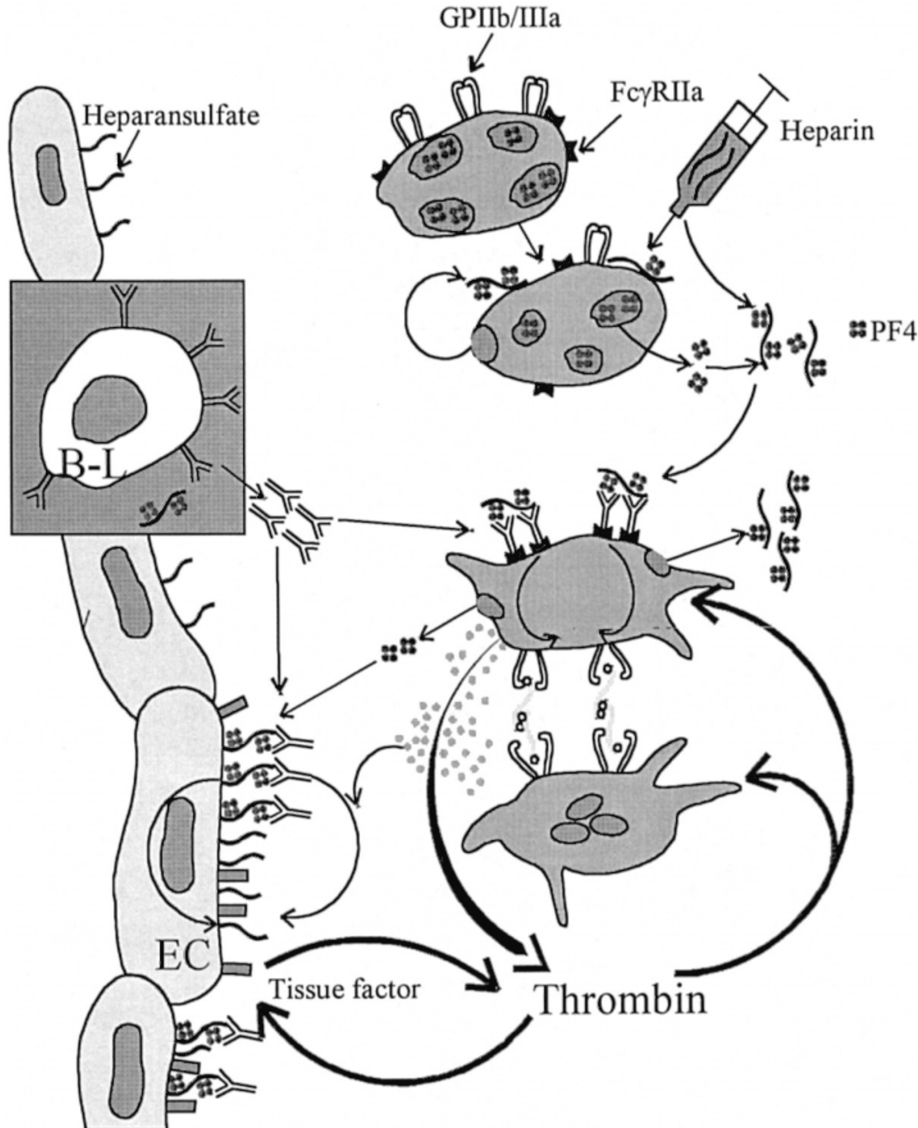


Fig. 39.4. Pathophysiology of heparin-induced thrombocytopenia[82]. Platelets activated by PF4/heparin–HIT–antibody complexes trigger a cascade of events that eventually lead to activation of the coagulation pathways, resulting in thrombin generation. The released PF4 also binds to endothelial cell heparan sulfate, forming antigen complexes to which HIT-antibodies can bind.

upregulate tissue factor expression. It is very likely that platelet microparticles and anti-PF4 heparin antibodies, known to be able to activate monocytes, also cause expression of tissue factor on monocytes[147].

Genetic polymorphisms of clotting factors causing enhanced thrombin generation such as Factor V Leiden[148] or prothrombin G20210A[149] or MTHFR C677T[149] do not seem to be risk factors for the development of clinical HIT.

Frequency of HIT

It is important to distinguish patients with HIT-antibodies only from patients with clinical HIT: studies screening symptomatic as well as asymptomatic patients[150–159] showed that most patients who form HIT antibodies do not develop clinical HIT. Clinical HIT however can occur in up to 3% of patients treated with unfractionated heparin[150]. Different patient populations can have greatly differing incidences of clinically inapparent HIT antibodies as well

Cambridge Books Online © Cambridge University Press, 2009

as clinically manifest HIT. Fig. 39.3 shows the cardiosurgical patients treated with UFH to be at highest risk of developing HIT-antibodies but associated thromboembolic complications are more frequent in patients undergoing orthopedic hip replacement surgery. Despite the fact that LMWHs cause HIT less often compared to UFH[150–160], this is only true within the same patient population: medical patients on UFH have a lower risk of developing HIT compared to orthopedic patients on LMWH.

Diagnosis of HIT

Clinical diagnosis

In HIT platelet counts usually drop by >50% or below 100 000 Gpt/l after day 5 of heparin treatment. In case of reexposure to heparin this drop can occur earlier[161,162]. Thrombocytopenia of <20 000 Gpt/l occurs in less than 10% of HIT-patients. Typical TECs associated with HIT are deep venous thrombosis, pulmonary embolism[163], venous limb gangrene (caused by temporary acquired protein C deficiency during initiation of coumarin anticoagulation)[143], cerebral vein thrombosis/adrenal hemorrhagic necrosis[164–166], arterial thrombosis,[119] heparin-induced skin lesions[167,168] and acute systemic reactions[169–171].

Differential diagnosis

Differential diagnoses include non-immune heparin associated thrombocytopenia, autoimmune thrombocytopenia, drug-dependent thrombocytopenias (see above), consumptive thrombohemorrhagic disorders like disseminated intravascular coagulopathy, post-transfusion purpura[172], and a variety of diseases accompanied by a substantial decrease of platelet counts[173]. In patients with adenocarcinoma, DIC can be triggered secondary to procoagulant material produced by neoplastic cells. This is typically enhanced when oral anticoagulation is started, most likely due to the induced protein C deficiency[168,173]. In severe pulmonary embolism, platelet activation by clot bound thrombin can result in a substantial decrease of platelet counts. Both syndromes can easily be misdiagnosed as HIT, because thrombocytopenia and the new thrombotic complication may occur after several days of heparin treatment. Although drug-dependent thrombocytopenia and post-transfusion purpura[172] occur with a time delay typical for HIT, they are characterized by very low platelet counts and are often associated with bleeding complications which would be atypical for HIT. Also, in clinical situations with acute platelet activation, the platelet count decrease occurs early and not with the time delay of 5–9 days as in HIT, i.e. in diabetic ketoacidosis, causing hyperaggregable platelets; in thrombolytic therapy potentially due to thrombin bound to fibrin split products; in infective endocarditis, by infection-related platelet activation sometimes accompanied by septic emboli; and in paroxysmal nocturnal hemoglobinuria, by platelet hypoproduction and complement mediated platelet destruction. Differentiation of antiphospholipid syndrome from HIT is sometimes difficult[174].

Laboratory testing

In vitro demonstration of HIT-antibodies by functional (or activation) and immunological assays[175] can help securing the clinical suspicion of HIT.

Activation assays are usually performed with washed donor platelets, incubated with heparin and patient serum. Whereas the serotonin release assay (SRA) is regarded the gold standard, it involves the need for radiolabels[176]. The heparin-induced platelet activation assay – while still technically demanding – is better suited for routine testing[177,178]. Other endpoints as luminography[179], platelet derived microparticle generation[180], or annexin V expression[181] may also be used. In the hands of the authors, results between these assays do not differ largely as long as washed platelets are used. However, sensitivity is reduced, if performed with platelet-rich plasma[182–185].

Antigen assays use surface-bound target antigen (PF4/heparin- or PF4/polyvinylsulfate-complexes, respectively) to detect the antibodies[91,96,186]. The fluid phase EIA for HIT antibodies[187] is a modification of the protein A antibody capture method[188]. The latter assay has several technical advantages such as lower background by removal of non-HIT IgG and less interaction of the microtitre plate surface with the antigen structure, but it only allows detection of antibodies of the IgG class. An assay using the microcolumn technique known from red blood cell serology for detection of HIT antibodies[189] is currently under evaluation.

Regardless which assay is used, it is important to incorporate clinical information in the interpretation of test results and to combine a sensitive functional assay and an antigen assay. Whereas functional and antigen assays have similar sensitivities for clinical HIT, antigen assays may have greater sensitivity for HIT antibody seroconversion. Thus the functional assays' positive predictive value for clinical HIT could be greater[153]. False negative results are estimated to be less than 5% if both test systems are applied.

Therapy

Immediate cessation of heparin is necessary when HIT develops. However, thrombin generation continues[190]. Up to 50% of patients with HIT who do not have a new

Cambridge Books Online © Cambridge University Press, 2009

HIT-associated thrombosis at the time HIT is clinically suspected on the basis of isolated thrombocytopenia will develop a new thrombotic complication during the next weeks[163,191–193]. Therefore, these patients should be treated with an alternative anticoagulant until recovery of platelet counts[194,195].

Prospective studies with lepirudin revealed that patients were at the highest risk of experiencing new thromboembolic complications in the acute phase of HIT despite cessation of heparin[192,193]. Thus, in cases of strong clinical suspicion of HIT, patients should immediately be treated with an alternative anticoagulant. Awaiting laboratory confirmation of HIT before alternative anticoagulation is started would cause a significant delay in most cases, enhancing the risk for the patient substantially.

There is clinical experience as well as approval by the authorities in several countries for the treatment of HIT with the direct thrombin inhibitors hirudin and argatroban as well as the heparinoid danaparoid.

Hirudin

Pharmacology

Unlike heparin, hirudin inhibits not only free but also clot-bound thrombin[196–199].

Terminal plasma elimination half-lifes ($t_{1/2}$ß) of 0.8–1.7 hours after intravenous (i.v.) injection of bolus lepirudin doses of 0.01–0.5 mg/kg, and 1.1–2.0 hours for continuous i.v. infusions over 6 hours are observed[200]. With subcutaneous (s.c.) administration, bioavailability is nearly 100%. After s.c. injection of 0.75 mg/kg, a peak lepirudin concentration of approximately 0.7 μg/ml occurs in 3 to 4 hours[201] (dosing schedules Table 39.6).

Renal clearance accounts for approximately 90% of the systemic clearance. The $t_{1/2}$ß of lepirudin is lengthened with deterioration of renal function[204–208]; in nephrectomized humans it is prolonged to up to 200 hours[209].

For monitoring, the aPTT[210,211] shows considerable variability between patients[212–215] and at higher hirudin plasma levels (aPTT values >70 seconds), the concentration-aPTT curve flattens and the correlation becomes poor[215–218]. A new tool for monitoring hirudin treatment is the ecarin clotting time (ECT)[219–222] especially in patients requiring a high hirudin dose, for example during cardiopulmonary bypass[223]. There is no antidote available for hirudin.

Clinical studies with hirudin in HIT

After a small pilot study[224] and several case reports showed the feasibility of lepirudin treatment in HIT patients[225–231], two prospective, multicentre, historically controlled trials, HAT-1 ($n = 82$)[192] and HAT-2 ($n = 112$)[193] were conducted in patients with laboratory confirmation of the clinical diagnosis of HIT.

Both studies had similar mortality rates (7.3% and 9.8%); however, limb amputations (3.7% and 8.9%) and new thromboembolic complications (9.8% and 17.9%) differed between the studies. All fatalities were judged to be due to the underlying disease, rather than use of lepirudin.

The combined endpoint (new TECs, limb amputation, death) at day 35 was 52.1% in a historical control ($n = 120$) and 25.4% in the HAT-1– ($P = 0.014$; adjusted risk ratio 0.508; 95% CI, 0.290–0.892) and 31.9% in the HAT-2 study ($P = 0.15$; adjusted risk ratio 0.709; 95% CI, 0.44–1.14).

In both studies there were more bleeding events in the lepirudin-treated group compared to controls (39.1% [HAT-1], 44.6% [HAT-2] vs. 27.2%), but not more bleedings requiring transfusion (9.9% [HAT-1], 12.9% [HAT-2] vs. 9.1%).

In a recent meta-analysis of both studies, patients with HIT and ongoing thrombosis treated with lepirudin showed a clear reduction of the combined endpoint compared to the historical control group ($P = 0.004$)[144]. Low aPTT ratios (1.0 to 1.5) were clearly subtherapeutic, with minimal effects on both clinical events and bleeding. In contrast, medium aPTT ratios (1.5 to 2.5) were associated with a pronounced reduction in clinical events (RR = 0.42) and a moderately increased risk of bleeding (RR = 3.21). Higher aPTT ratios (>2.5) did not further reduce clinical events, but doubled the risk ratio of bleeding (RR = 6.03). Thus, an aPTT ratio of 1.5 to 2.5 is the recommended target range[144].

Whereas a change in the prothrombin time at the start of treatment with lepirudin is of minor clinical relevance, no evidence was found indicating that cessation of lepirudin causes a change of the INRs[144].

Induction of antibodies

As a non-human protein, recombinant hirudin has immunogenic properties that can cause antihirudin antibody formation[232–235]. Of 198 HIT patients treated for ≥5 days with lepirudin, 45% developed antihirudin antibodies of the immunoglobulin G (IgG) class[232]. In 2–3% of patients with antihirudin antibodies, the hirudin dose had to be decreased by more than 60% to maintain the aPTT within the target range[232]. This enhanced anticoagulant effect may be caused by decreased renal elimination of the hirudin–antihirudin complexes. In a few patients, development of antihirudin antibodies was paralleled by a decrease of the aPTT.

Cambridge Books Online © Cambridge University Press, 2009

Table 39.6. Dosing schedules tested in hirudin studies (data were generated with lepirudin unless otherwise indicated)[195]

	HIT and thrombosis[b](c)	HIT with thrombosis and concomitant thrombolysis(c)	HIT with isolated thrombocyto-penia (c)	Thrombosis prophylaxis in patients with a history of HIT[d](e,a)	Renal dialysis alternate days (d)	Renal dialysis or CVVH in intensive care unit patients (e)	Cardiopulmonary bypass surgery (e)	Unstable angina (a)	PTCA (b)	Acute MI (a)
Bolus[c]	0.4 mg/kg b.w. i.v	0.2 mg/kg b.w. i.v			0.08–0.1 mg/kg b.w. i.v. predialysis		0.25 mg/kg b.w. i.v.; 0.2 mg/kg b.w. in the priming fluid of the HLM	0.4 mg/kg b.w. i.v	0.3 mg/kg i.v. or 0.5 mg/kg i.v.	0.2 mg/kg i.v. or 0.1 mg/kg i.v
i.v. infusion[c]	0.15 mg/kg b.w./h	0.1 mg/kg b.w./h	0.1 mg/kg b.w./h	0.1 mg/kg b.w./h		0.005 mg/kg b.w./h	0.5 mg/min[e]	0.15 mg/kg b.w./h	0.12 mg/kg/h for 24 h or 0.24 mg/kg/h for 24 h; followed by 0.04 mg/kg/h for 24 h	0.5 mg/kg b.w. bid s.c. or 0.1 mg/kg b.w./h
Target aPTT ratio[a]	1.5–2.5	1.5–2.5	1.5–2.0	1.5–2.0	2.0–3.5	1.5–2.5	Monitored by ECT: >2.5 μg/ml before start of HLM; 3.5–4.5 μg/ml during CPB	aPTT 60–100 s		1.5–2.0

Notes:

Due to lack of systematic data, hirudin cannot be recommended during pregnancy and lactation or in children as long as other treatment options are available.

During concomitant treatment with aspirin, GPIIb/IIIa inhibitors, fibrinolysis, or in patients with platelet counts <100 000/μl, the bleeding risk is increased and dosage may have to be reduced.

In patients with renal impairment, dosage must be reduced to avoid overdosage.

Abbreviations: aPTT = activated partial thromboplastin time; HIT = heparin-induced thrombocytopenia; TEC = thromboembolic complication; i.v. = intravenous; s.c. = subcutaneous; b.w. = body weight; CVVH = chronic-veno-venous hemofiltration; ECT = ecarin clotting time; HLM = heart–lung machine; CPB = cardiopulmonary bypass; bid = two times daily.

[a] If Actin FS or Neothromtin reagents are used in Europe, different ranges may apply (e.g., 1.5–3.0).

[b] Approved indication in the EU and the USA reduce bolus by 50% in elderly patients (compensated renal insufficiency).

[c] Maximum body weight for calculation of dosage: 110 kg.

[d] Desirudin has been shown to be effective in a dosage of 15 mg s.c. bid following orthopedic hip replacement surgery (d).[202,203]

[e] Stop 15 min before end of CPB. Put 5 mg lepirudin into CPB-machine after disconnection to avoid clotting.

Levels of evidence for the recommendations:

(a) prospective randomized double-blind trials.

(b) prospective randomized open trial.

(c) uncontrolled (due to ethical reasons), prospective multicentre trials.

(d) crossover study, prospective dose escalation trial.

(e) case observations.

Cambridge Books Online © Cambridge University Press, 2009

Argatroban

Pharmacology

Argatroban is a small (anhydrous, MW 508.7) synthetic direct thrombin inhibitor that specifically and reversibly binds to the catalytic site region of thrombin[236].

In healthy volunteers the pharmacodynamic steady-state was reached after 161–268 minutes in doses from 1.25 to 10 μg/kg/min without bolus and 150–268 minutes with a bolus of 250 μg/kg.

The main route of elimination is hepatic metabolization, the pharmacodynamic half- life is 36 minutes and severe renal impairment does not affect the elimination of argatroban (at a dose of 5.0 μg/kg/min for 4 hours). This is a major advantage in HIT patients with renal impairment in whom quickly adjustable anticoagulation is required.

Initial dosing in adults without hepatic impairment is 2 μg/kg/min as a continuous intravenous infusion. Steady state levels are typically reached after about 2.5 hours. Monitoring using the aPTT should be started at that time aiming at 1.5–3.0 times baseline values, not to exceed 100 seconds. Adjusted doses should be lower than 10 μg/kg/minute.

There are combined effects of oral anticoagulants and argatroban on the INR, causing artificially increased INR values. Therefore, INR values should be monitored daily. No loading dose of the oral anticoagulant should be given, instead therapy should be started with the expected daily dose. With doses of argatroban of up to 2 μg/kg/min, argatroban can be discontinued once the INR is >4. INR should then be checked every 4–6 hours. If a repeat INR is below the therapeutic range, the argatroban infusion should be resumed and the procedure repeated daily until the therapeutic INR level is reached on oral anticoagulation alone. With oral anticoagulation and argatroban doses >2 μg/kg/min, dosing should be temporarily reduced to 2 μg/kg/min and the INR checked 4–6 hours later.

Clinical studies with argatroban in HIT

Argatroban has been assessed in a prospective trial in patients with HIT with a similar design as the lepirudin studies. A major difference to the lepirudin studies was that patients with a clinical suspicion of HIT only were also included. In brief argatroban was associated with a lower composite event rate (death, limb amputation, new thrombosis) compared to historic control subjects (patients with isolated HIT 25.6 vs. 38.8%, $P = 0.014$ and patients with HIT and thrombosis 43.8 vs. 56.5%, $P < 0.130$)[237]. Significant between-group differences by time-to-event analysis of the composite end point favoured argatroban treatment in HIT ($P = 0.010$) and HIT and thrombosis ($P = 0.014$).

Danaparoid

Pharmacology

The heparinoid danaparoid-sodium[238–240] consists of 84% heparan sulfate, 12% dermatan sulfate and 4% chondroitin sulfate[241].

Danaparoid mainly exhibits anti-Xa activity, with a half life of approx. 24 hours. Its antithrombin activity has a half-life of about 2–4 hours. Danaparoid is mainly eliminated renally[242]. In patients with renal failure a dose reduction of about 30% is required to maintain aFXa levels within the therapeutic range[115].

Danaparoid does not significantly prolong aPTT, activated clotting time or INR, thus anti-FXa-activity has to be used for monitoring. It is important to use a danaparoid-calibration curve[243].

Bioavailability is nearly 100% after s.c. injection and plasma levels are usually predictable. Monitoring is recommended in substantial renal impairment, unusually high or low body weight, life- or limb-threatening thrombosis, unexpected bleeding and critically ill or unstable patients[115].

Clinical studies with danaparoid in HIT

Danaparoid has been assessed in a compassionate use programme and one prospective, randomized clinical trial. In the named patient programme, 667 HIT patients with 708 treatment episodes were included[239] (for doses see Table 39.7). The treating physicians judged treatment to be successful in 93% because of platelet count recovery or clinical improvement of patients. In 1.7% of patients, new thromboembolic complications occurred. Of 114 (17.1%) deaths, 14 (12.2%) were thought to be related to the danaparoid treatment.

In a prospective, randomized controlled study comparing danaparoid plus warfarin with dextran 70 plus warfarin, 42 HIT patients with acute thrombosis were stratified depending on the severity of their thrombosis[240]. The treatment consisted of a danaparoid 2400 IU bolus, followed by 400 IU/h for 2 hours, then 300 IU/h for 2 hours and then 200 IU/h for 5 days. In patients with 'mild' thrombosis improvement was seen in a slightly higher proportion of the danaparoid-treated patients (92% vs. 71%, non-significant). A significant difference in favor of the danaparoid-treated patients was seen in the group with 'severe' thrombosis: 85% vs. 30%, $P = 0.03$.

Cross-reactivity with HIT antibodies

Danaparoid cross-reacts with HIT-antibodies in vitro at a rate of 7–50%, depending on the sensitivity of the assay used[115,187]. Despite anecdotal reports of unfavourable out-

Cambridge Books Online © Cambridge University Press, 2009

comes[244], two studies found no difference in clinical outcomes comparing HIT patients treated with danaparoid with or without in vitro cross-reactivity[187,245]. It therefore seems reasonable to limit testing for in vitro cross-reactions to patients who develop new, progressive or recurrent thrombocytopenia or thrombosis during treatment with danaparoid[115]. Should a positive result occur in such a patient, anticoagulation should be switched to a different drug[246].

Clinical experiences with treatment of HIT in special circumstances

Dialysis

The feasibility of hirudin for hemodialysis has been shown in small patient groups[205–208,247,248] (Table 39.7). In critically ill patients, a reduced dosage is often sufficient to enable hemodialysis or hemofiltration (continuous i.v. infusion of 0.005 mg/kg/h immediately before the filter, adjusted to ECT or to aPTT)[249].

Argatroban has been used successfully in hemodialysis[250], but systematic data are lacking at present.

Danaparoid has also been successfully used in intermittent as well as in continuous hemodialysis[115,251,252] (Table 39.7).

Extracorporeal circulation

In patients undergoing cardiac surgery with lepirudin[223,253,254,255], close bedside monitoring using the ECT is essential[217,218,223,255–258].

A pre-CPB lepirudin bolus of 0.25 mg/kg should be given and 0.2 mg/kg added to the priming solution. Plasma levels at the start of CPB should be >2.5 μg/ml (if lower, an additional 10 mg bolus should be given). During CPB, 0.5 mg/min should be infused continuously, and adjusted using ECT measurements every 15 min (target range 3.5–4.5 μg/ml). The infusion should be stopped 15 min before the anticipated end of CPB. Patients with impaired renal function usually require hemofiltration to reduce lepirudin plasma levels, but are still at high risk of developing bleeding complications[223].

Experiences with danaparoid and CPB in 47 patients[259] were judged successful in 95.7% at the time of postoperative wound closure. Because of clotting as well as bleeding problems in this and in other studies using a fixed danaparoid dose regimen, Pötzsch and Madlener[223] developed an algorithm using dose adjustments according to aFXa-measurements intraoperatively: 3000 aFXaU should be added to the priming solution with a bolus of 100 aFXaU/kg given to the patient 15–20 min before start of CPB. aFXa target level is >1.5 U/ml. During CPB a danaparoid infusion of 200 U/h should be started with a target range of 1.2–1.8 U/ml. aFXa activity monitoring every 15 min is mandatory. Thirty minutes before anticipated end of CPB, the infusion should be stopped.

In patients with a history of HIT and no circulating HIT-antibodies, short-term re-exposure with heparin is probably a safer treatment alternative (see below)[260,261].

Children

Schiffmann et al.[225] described a 12-year-old patient with HIT and multiple TECs. Using a bolus of 0.2 mg/kg lepirudin followed by a continuous infusion of 0.1–0.7 mg/kg/h, the aPTT was prolonged to 45–85 s. Planned surgical interventions were possible after discontinuing lepirudin for 4 hours.

Danaparoid has been used in children successfully[262]. For thrombosis prophylaxis in children below 55 kg, the manufacturer[263] recommends subcutaneous injections of 10 aFXaU/kg bid, in acute thrombosis a bolus of 30 aFXaU/kg followed by 1.2–4.0 aFXaU/kg/h with a target range of 0.4–0.6 aFXaU/ml or 0.5–0.8 aFXaU/ml, respectively.

Pregnancy

In rabbits, the hirudin plasma concentration in the fetuses was 1/60 of the respective maternal concentration[264]. Embryotoxic effects were seen at high, but not at low doses in rabbits (30 mg/kg/day and 1 and 10 mg/kg/day, respectively)[200].

Subcutaneous r-hirudin (15 mg twice daily) was used successfully from week 25 in a pregnant woman with HIT and cross-reactivity to danaparoid[265]. Lindhoff-Last et al.[266] detected no hirudin in the breast milk of a woman treated with lepirudin 50 mg s.c. bid.

Danaparoid has been used successfully in therapeutic and prophylactic doses in pregnancy[115]. Studying aFXa activities in maternal and placental blood, no evidence was found for danaparoid crossing the placenta[267].

Adjunctive treatments

Adjunctive treatments in HIT have not been assessed systematically. They may be considered as additional treatment options in individual patients[195].

Regional or systemic thrombolysis should be considered in patients with limb-threatening thrombosis or pulmonary embolism with severe cardiovascular compromise; surgical thrombectomy for limb-threatening large-vessel arterial thromboembolism is not contraindicated, even in thrombocytopenic patients. Intravenous gammaglobulin can be used in selected patients to block Fc-receptor dependent platelet activating effects of HIT antibodies.

Cambridge Books Online © Cambridge University Press, 2009

Table 39.7. Danaparoid dosing schedules[115]

Clinical indication	Danaparoid dosing schedule
Venous thromboembolism: prophylaxis	750 U s.c. bid or tid
Venous thromboembolism: treatment	2250 U i.v. bolus[a], followed by 400 U/h for 4 h, 300 U/h for 4 h, then 150–200 U/h for ≥5 days, aiming at a plasma anti-Xa level of 0.5–0.8 U/ml *Subcutaneous administration schedule*: 1500–2250 U s.c. bid (bioavailability is almost 100% when given by s.c. injections; thus, 2250 U s.c. bid is approximately equal to an i.v. infusion rate of 200 U/h (4500 U/24 h vs. 4800 U/24 h, respectively))
Arterial thromboembolism: treatment	See venous thromboembolism treatment schedule
Embolectomy or other peripheral vascular surgery	*Preoperative*: 2250 U i.v. bolus[a]; *intraoperative flushes*: 750 U in 250 ml saline, using up to 50 ml; *postoperative*: 750 U s.c. tid (low-risk) or 150–200 U/h (high-risk) beginning at least 6 h after surgery
Hemodialysis (on alternate days)	3750 U i.v. before 1st and 2nd dialyses; 3000 U for 3rd dialysis; then 2250 U for subsequent dialyses, aiming at plasma anti-Xa level of <0.3 U/ml before each dialysis and 0.5 –0.8 U/ml during dialysis
Hemofiltration	2250 U i.v. bolus, followed by 600 U/h for 4h, then 400 U/h for 4 h, then 200–400 U/h aiming at a plasma anti-Xa level of 0.5–1.0 U/ml
Cardiopulmonary bypass surgery (CPB)	125 U/kg i.v. bolus after thoracotomy 3 U/ml in priming fluid of CPB apparatus 7 U/kg/h i.v. infusion commencing after bypass hookup, and continued until 45 min prior to expectation of stopping bypass
Cardiac catheterization	*Preprocedure*: 2250 U i.v. bolus (3000 U if 75–90 kg and 3750 U if >90 kg)
Percutaneous transluminal coronary angioplasty (PTCA) or intra-aortic balloon pump	*Preprocedure*: bolus as per above row; *post-procedure*: 150–200 U/hr for 1–2 days after PTCA (or until removal of balloon pump)
Catheter patency	750 U in 50 ml saline, then 5–10 ml per port, or as required
Pediatric dosage considerations:	*Prophylaxis*: 10 U/kg s.c. bid *Treatment:* 30 U/kg b.w., then 1.2–2.0 U/kg b.w./h depending upon severity of thrombosis

Notes:
[a] Adjust i.v. danaparoid bolus for body weight: <60 kg, 1500 U; 60–75 kg, 2250 U; 75–90 kg, 3000 U; >90 kg, 3750 U.

Plasmapheresis is only recommended when acquired deficiency of one or more natural anticoagulant proteins is suspected. Antiplatelet agents such as aspirin might be beneficial in patients with HIT and an increased risk for arterial thromboembolism, enhancing bleeding risk as well. Platelet glycoprotein IIb/IIIa inhibitors should be considered as experimental in this clinical setting.

Re-exposure of patients with a history of HIT to heparin

Even though not all patients with a history of HIT show an anamnestic response when reexposed to heparin[161], both UFH and LMWH should be avoided in patients with a history of HIT. Short-term re-exposure may be considered in special clinical settings (such as need for cardiopulmonary bypass) after a careful risk/benefit analysis.

Warkentin and Kelton[161], in a study including 260 patients, analyzed the timing of onset of HIT in relation to a recent (≤100 days) or remote (>100 days) prior heparin use. Two patterns were observed: 77 patients developed immediate onset of HIT with a platelet count fall within 36 h after starting heparin. All 77 patients had received heparin within the past 100 days. In a similar study we also identified heparin re-exposure within 3 months as a risk factor for early onset of HIT[162].

This temporal pattern may offer treatment options in patients with a history of HIT who are in need of surgery necessitating CPB. Provided no circulating HIT-antibodies are detectable, these patients can receive heparin during CPB if heparin exposure is strictly avoided pre- and post-operatively. With this strategy, complications using anticoagulants without an antidote during CPB, i.e. hirudin, argatroban or danaparoid, can be avoided[223,261,268].

Cambridge Books Online © Cambridge University Press, 2009

References

1 Salama A, Mueller-Eckhardt C. On the mechanisms of sensitization and attachment of antibodies to RBC in drug-induced immune hemolytic anemia. *Blood* 1987; 69: 1006–1010.
2 Stroncek DF. Drug-induced immune neutropenia. *Transfus Med Rev* 1993; 7: 268–274.
3 Bux J, Ernst-Schlegel M, Rothe B, Panzer C. Neutropenia and anaemia due to carbimazole-dependent antibodies. *Br J Haematol* 2000; 109: 243–247.
4 von dem Borne AE, Pegels JG, van der Stadt RJ, van der Plas-van Dalen CM, Helmerhorst FM. Thrombocytopenia associated with gold therapy: a drug-induced autoimmune disease? *Br J Haematol* 1986; 63: 509–516.
5 George JN, Raskob GE, Rizvi Shah S et al. Drug-induced thrombocytopenia: a systematic review of published case reports. *Ann Intern Med* 1998; 129: 886–890.
6 Kiefel V, Mueller-Eckhardt C. Drug-induced immune hemocytopenias (German). *Dtsch Med Wochenschr* 1993; 118: 113–118.
7 Aster RH: Drug-induced immune thrombocytopenia: an overview of pathogenesis. *Semin Hematol* 1999; 36: 2–6.
8 Ackroyd JF. Drug-induced thrombocytopenia. An immunological phenomenon. *Vox Sang* 1983; 45: 257–259.
9 Murphy MF, Riordan T, Minchinton RM et al. Demonstration of an immune-mediated mechanism of penicillin-induced neutropenia and thrombocytopenia. *Br J Haematol* 1983; 55: 155–160.
10 Salamon DJ, Nusbacher J, Stroupe T, Wilson JH, Hanrahan JB. Red cell and platelet-bound IgG penicillin antibodies in a patient with thrombocytopenia. *Transfusion* 1984; 24: 395–398.
11 Christie DJ, Aster RH. Drug-antibody-platelet interaction in quinine- and quinidine-induced thrombocytopenia. *J Clin Invest* 1982; 70: 989–998.
12 Kiefel V, Santoso S, Schmidt S, Salama A, Mueller-Eckhardt-C. Metabolite-specific (IgG) and drug-specific antibodies (IgG, IgM) in two cases of trimethoprim-sulfamethoxazole-induced immune thrombocytopenia. *Transfusion* 1987; 27: 262–265.
13 Shulman NR, Reid DM. Mechanisms of drug-induced immunologically mediated cytopenias. *Transfus Med Rev* 1993; 7: 215–229.
14 Miescher PA, Miescher A. Immunologic drug-induced blood dyscrasias (German). *Klin Wochenschr* 1978; 56: 1–5.
15 Christie DJ, Mullen PC, Aster RH. Fab-mediated binding of drug dependent antibodies to platelets in quinidine- and quinine-induced thrombocytopenia. *J Clin Invest* 1985; 75: 310–314.
16 Smith ME, Reid DM, Jones CE, Jordan JV, Kautz CA, Shulman NR. Binding of quinine- and quinidine-dependent drug antibodies to platelets is mediated by the Fab domain of the immunoglobulin G and is not Fc dependent. *J Clin Invest* 1987; 79: 912–917.
17 Kunicki TJ, Johnson MM, Aster RH. Absence of the platelet receptor for drug dependent antibodies in the Bernard–Soulier Syndrome. *J Clin Invest* 1978; 62: 716–719.
18 van Leeuwen EF, Engelfriet CP, von dem Borne AEGK. Studies on quinine- and quinidine-dependent antibodies against platelets and their reaction with platelets in the Bernard-Soulier Syndrome. *Br J Haematol* 1982; 51: 551–560.
19 Berndt MC, Chong BH, Bull HA, Zola H, Castaldi PA. Molecular characterization of quinine/quinidine drug-dependent antibody platelet interaction using monoclonal antibodies. *Blood* 1985; 66: 1292–1301.
20 Stricker RB, Shuman MA. Quinidine purpura: evidence that glycoprotein V is a target platelet antigen. *Blood* 1986; 67: 1377–1381.
21 Christie DJ, Aster RH. Quinine- and quinidine platelet antibodies can react with GP IIb/IIIa. *Br J Haematol* 1987; 67: 213–219.
22 Christie DJ. Specificity of drug-induced immune cytopenias. *Transfus Med Rev* 1993; 7: 230–241.
23 Pfueller SL, Bilston RA, Jane S, Gibson J. Expression of the drug-dependent antigen for quinine-dependent antiplatelet antibodies on GP IIIa but not that on GP Ib, IIb or IX on human endothelial cells. *Thromb Haemost* 1990; 63: 279–281.
24 Visentin GP, Newman PJ, Aster RH. Characteristics of quinine- and quinidine induced antibodies specific for platelet glycoproteins IIb and IIIa. *Blood* 1991; 77: 2668–2676.
25 Chong BH, Du XP, Berndt MC, Horn S, Chesterman CN. Characterization of the binding domains on platelet glycoproteins Ib–IX and IIb/IIIa complexes for the quinine/quinidine-dependent antibodies. *Blood* 1991; 77: 2190–2199.
26 Kroll H, Sun QH, Santoso S. Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a target glycoprotein in drug-induced thrombocytopenia. *Blood* 2000; 96: 1409–1414.
27 George JN, Berkowitz SD, Raskob GE. Platelets: acute thrombocytopenia. *Hematology* 1998; (Suppl): 371–383.
28 Aster RH, George JN. Thrombocytopenia due to enhanced platelet destruction by immunologic mechanisms. In: Williams WJ, Beutler E, Erslev AJ, Lichtman MA, eds. *Hematology*. New York: McGraw-Hill; 1990: 1370–1398.
29 Eisner EV, Kasper K. Immune thrombocytopenia due to a metabolite of para-aminosalicylic acid. *Am J Med* 1972; 53: 790–796.
30 Eisner EV, Shahidi NT. Immune thrombocytopenia due to a drug metabolite. *N Engl J Med* 1972; 287: 376–381.
31 Meyer T, Herrmann C, Wiegand V, Mathias B, Kiefel V, Mueller-Eckhardt C. Immune thrombocytopenia associated with hemorrhagic diathesis due to ibuprofen administration. *Clin Invest* 1993; 71: 413–415.
32 Kiefel V. Differential diagnosis of acute thrombocytopenia. pp17–41. In: Warkentin TE, Greinacher A. *Heparin-induced thrombocytopenia*. New York, Basel: Marcel Dekker, Inc. 2001, 2nd edn.
33 McFarland JG. Laboratory investigation of drug-induced immune thrombocytopenias. *Trans Med Rev* 1993; 7: 275–287.

Cambridge Books Online © Cambridge University Press, 2009

34 Kiefel V, Jaeger S, Mueller-Eckhardt C. Competitive enzyme-linked immunoassay for the quantitation of platelet associated immunoglobulins (IgG, IgM, IgA) and complement (C3c, C3d). *Vox Sang* 1987; 53: 151–156.

35 Kiefel V. The MAIPA assay and its applications in immunohematology. *Transfus Med* 1992; 2: 181–188.

36 Visentin GP, Wolfmeyer K, Newman PJ, Aster RH. Detection of drug-dependent, platelet-reactive antibodies by antigen-capture ELISA and flow cytometry. *Transfusion* 1990; 30: 694–700.

37 Pfueller SL, Bilston RA, Logan D et al. Heterogeneity of drug-dependent platelet antigens and their antibodies in quinine- and quinidine-induced thrombocytopenia: involvement of glycoproteins Ib, IIb, IIa and IX. *Blood* 1988; 72: 1155–1162.

38 Rizvi MA, Shah RS, Raskob GE, George JN. Drug-induced thrombocytopenia. *Curr Opin in Hematol* 1999; 6: 349–353.

39 Jordan R, Mascelli MA. Pharmacological differentation of GP IIb/IIIa inhibitors. *Eur Heart J Suppl* 1999; 1: E3–E10.

40 EPIC Investigators. Use of a monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high-risk coronary angioplasty. *N Engl J Med* 1994; 330: 956–961.

41 EPILOG Investigators. Platelet glycoprotein IIb/IIIa receptor blockade and low-dose heparin during percutaneous coronary revascularization. *N Engl J Med* 1997; 336: 1689–1696.

42 CAPTURE Investigators. Randomised placebo-controlled trial of abciximab before and during coronary intervention in refractory unstable angina: The CAPTURE study. *Lancet* 1997; 349: 1429–1435.

43 IMPACT Investigators. Multicenter, randomised, double-blind, placebo-controlled trial of the platelet integrin glycoprotein IIb/IIIa blocker Integrilin in elective coronary intervention. *Circulation* 1995; 91: 2151–2157.

44 IMPACT-AMI Investigators. Combined accelerated tissue-plasminogen activator and platelet glycoprotein IIb/IIIa integrin receptor blockade with Integrilin in acute myocardial infarction. Results of a randomised, placebo-controlled, dose ranging trial. *Circulation* 1997; 95: 846–854.

45 PURSUIT Investigators. Inhibition of platelet glycoprotein IIb/IIIa with eptifibatide in patients with acute coronary syndromes. *N Engl J Med* 1998; 339: 436–443.

46 RESTORE Investigators. Effects of platelet glycoprotein IIb/IIIa blockade with tirofiban on adverse cardiac events in patients with unstable angina or acute myocardial infarction undergoing coronary angioplasty. *Circulation* 1997; 96: 1445–1453.

47 Platelet Receptor Inhibition of Ischemic Syndrome Management (PRISM) Study Investigators. Comparison of aspirin plus tirofiban with aspirin plus heparin for unstable angina. *N Engl J Med* 1998; 338: 1498–1505.

48 Platelet Receptor Inhibition of Ischemic Syndrome Management in Patients Limited by Unstable Signs and Symptoms (PRISM-PLUS) Study Investigators. Inhibition of the platelet glycoprotein IIb/IIIa receptor with tirofiban in unstable angina and non-Q-wave myocardial infarction. *N Engl J Med* 1998; 338: 1488–1497.

49 Pinton P. Abciximab-induced thrombocytopenia during treatment of acute coronary syndromes by angioplasty [French]. *Ann Cardiol Angiol* 1998; 47: 351–358.

50 Berkowitz SD, Harrington RA, Rund MM, Tcheng JE. Acute profound thrombocytopenia after c7E3 Fab (abciximab) therapy. *Circulation* 1997; 95: 809–813.

51 Berkowitz SD, Harrington RA, Rund MM. Acute profound thrombocytopenia after c7E3 Fab therapy. [Reply letter] *Circulation* 1997; 96: 3810.

52 Berkowitz SD, Sane DC, Sigmon KN et al. Occurrence and clinical significance of thrombocytopenia in patients undergoing high-risk percutaneous coronary revascularisation. *J Am Coll Cardiol* 1998; 32: 311–319.

53 Kereiakes DJ, Essell JH, Abbotsmith CW, Broderick TM, Runyon JP. Abciximab-associated profound thrombocytopenia. Therapy with immunoglobulin and platelet transfusion. *Am J Cardiol* 1996; 78: 1161–1163.

54 Jubelirer SJ, Koenig BA, Bates MC. Acute profound thrombocytopenia following c7E3 Fab (Abciximab) therapy: case reports, review of the literature and implications for therapy. *Am J Hematol* 1999; 61: 205–208.

55 Mascelli MA, Lance ET, Damaraju L, Wagner CL, Weisman HF, Jordan RE. Pharmacodynamic profile of short-term abciximab treatment demonstrates prolonged platelet inhibition with gradual recovery from GP IIb/IIIa receptor blockade. *Circulation* 1998; 97: 1680–1688.

56 Nurden P, Poujol C, Durrieu-Jais C et al. Labeling of the internal pool of GP IIb–IIIa in platelets by c7E3 Fab fragments (abciximab): flow and endocytic mechanisms contribute to the transport. *Blood* 1999; 93: 1622–1633.

57 Moll S, Poepping I, Hauck S, Gulba D, Dietz R: Pseudothrombocytopenia after abciximab (Reopro) treatment. *Circulation* 1999; 100: 1460.

58 Holmes MB, Kabbani S, Watkins MW, Battle RW, Schneider DJ. Images in cardiovascular medicine. Abciximab-associated pseudothrombocytopenia. *Circulation* 2000; 101: 938–939.

59 Kozak M, Dovc T, Rozman P, Blinc A. A case of pseudothrombocytopenia after infusion of abciximab in vivo and anticoagulant-independent platelet clumping after rechallenge with abciximab in vitro. *Wien Klin Wochenschr* 2000; 112: 138–141.

60 Sane DC, Damaraju LV, Topol EJ et al. Occurrence and clinical significance of pseudothrombocytopenia during Abciximab therapy. *J Am Col Cardiol* 2000; 36: 75–83.

61 Jennings LK, White MM. Expression of ligand-induced binding sites on glycoprotein IIb/IIIa complexes and the effect of various inhibitors. *Am Heart J* 1998; 135: 179–183.

62 Madan M, Berkowitz SD. Understanding thrombocytopenia and antigenicity with glycoprotein IIb–IIIa inhibitors. *Am Heart J* 1999; 138: 317–326.

63 Peter K, Straub A, Kohler B et al. Platelet activation as a potential mechanism of GP IIb/IIIa inhibitor-induced thrombocytopenia. *Am J Cardiol* 1999; 84: 519–524.

64 Matzdorff AC, Kuhnel G, Kemkes-Matthes B, Pralle H, Voss R, Fareed J. Effect of glycoprotein IIb/IIIa inhibitors on CD62p

Cambridge Books Online © Cambridge University Press, 2009

expression, platelet aggregates, and microparticles in vitro. *J Lab Clin Med* 2000; 135: 247–255.

65 Cines DB. Glycoprotein IIb/IIIa antagonists: Potential induction and detection of drug-dependent antiplatelet antibodies. *Am Heart J* 1998; 135: 152–159.

66 Curtis BR, McFarland JG, Garrity MM, Aster RH. Thrombocytopenia induced by Abciximab: studies of pathogenesis. *Blood* 1998; (Suppl): Abstract 733.

67 Kecskes M, Stockelberg D, Grip L, Kutti J, Wadenvik H. Reopro induced severe thrombocytopenia. *Blood* 1998; (Suppl): Abstract 726.

68 Kroll H, Bresgen L, Giptner A. Thrombocytopenia due to the fibrinogen receptor antagonist abciximab may be caused by drug-dependent antibodies. *Ann Hematol* 2000; 79(Suppl): Abstract V197.

69 Kleiman NS, Tcheng JE. Safety issues surrounding use of platelet GP IIb/IIIa antagonists: reversibility and readministration. *Eur Heart J* Suppls 1999; 1(Suppl.E): E36–E42.

70 Tcheng JE, Kereiakes DJ, Braden GA et al. Safety of abciximab retreatment-final clinical report of the Reopro® readministration registry (R3). *Circulation* 1998; (Suppl.17): 1–17.

71 Weismann RE, Tobin RW. Arterial embolism occurring during systemic heparin therapy. *Arch Surg* 1958; 76: 219–227.

72 Sobel M, Adelman B. Characterization of platelet binding of heparins and other glycosaminoglycans. *Thromb Res* 1988; 50: 815–826.

73 Horne MK III. Heparin binding to normal and abnormal platelets. *Thromb Res* 1988; 51: 135–144.

74 Horne MK III, Chao ES. Heparin binding to resting and activated platelets. *Blood* 1989; 74: 238–243.

75 Horne III MK. Nonimmune heparin-platelet interactions: implications for the pathogenesis of heparin-induced thrombocytopenia. In Warkentin TE and Greinacher A. eds. *Heparin-induced thrombocytopenia*, New York: Marcel Dekker, 2001, 2nd ed.: 123–136.

76 Horne MK III, Chao ES. The effect of molecular weight on heparin binding to platelets. *Br J Haematol* 1990; 74: 306–312.

77 Greinacher A, Michels I, Liebenhoff U, Presek P, Mueller-Eckhardt C. Heparin-associated thrombocytopenia: immune complexes are attached to the platelet membrane by the negative charge of highly sulphated oligosaccharides. *Br J Haematol* 1993; 84: 711–716.

78 Salzman EW, Rosenberg RD, Smith MH, Lindon JN, Favreau L. Effect of heparin and heparin fractions on platelet aggregation. *J Clin Invest* 1980; 65: 64–73.

79 Chong BH, Ismail F. The mechanism of heparin-induced platelet aggregation. *Eur J Haematol* 1989; 43: 245–251.

80 Brace LD, Fareed J. Heparin-induced platelet aggregation. II. Dose/response relationships for two low molecular weight heparin fractions (CY 216 and CY 222). *Thromb Res* 1990; 59: 1–14.

81 Burgess JK, Chong BH. The platelet proaggregating and potentiating effects of unfractionated heparin, low molecular weight heparin and heparinoid in intensive care patients and healthy controls. *Eur J Haematol* 1997; 58: 279–285.

82 Warkentin TE, Chong BH, Greinacher A. Heparin-induced thrombocytopenia: towards consensus. *Thromb Haemost* 1998; 79: 1–7.

83 Chong BH. Heparin-induced thrombocytopenia. *Blood Rev* 1988; 2: 108–114.

84 Warkentin TE, Kelton JG. Interaction of heparin with platelets, including heparin-induced thrombocytopenia. In: Bounameaux H, ed. *Low-molecular-weight heparins in prophylaxis and therapy of thromboembolic diseases.* New York: Marcel Dekker; 1994: 75–127.

85 Mikhailidis DP, Barradas MA, Jeremy JY, Gracey L, Wakeling A, Dandona P. Heparin-induced platelet aggregation in anorexia nervosa and in severe peripheral vascular disease. *Eur J Clin Invest* 1985; 15: 313–319.

86 Reininger CB, Greinacher A, Graf J, Lasser R, Steckmeier B, Schweiberer L. Platelets of patients with peripheral arterial disease are hypersensitive to heparin. *Thromb Res* 1996; 81: 641–649.

87 Greinacher A. Antigen generation in heparin-associated thrombocytopenia: the nonimmunologic type and the immunologic type are closely linked in their pathogenesis. *Semin Thromb Haemost* 1995; 21: 106–116.

88 Horne III MK, Hutchison KJ. Simultaneous binding of heparin and platelet factor-4 to platelets: further insights into the mechanism of heparin-induced thrombocytopenia. *Am J Hematol* 1998; 58: 24–30.

89 Lane DA, Denton J, Flynn AM, Thunberg L, Lindahl U. Anticoagulant activities of heparin oligosaccharides and their neutralization by platelet factor 4. *Biochem J* 1984; 218: 725–732.

90 Bock PE, Luscombe M, Marshall SE, Pepper DS, Holbrook JJ. The multiple complexes formed by the interaction of platelet factor 4 with heparin. *Biochem J* 1980; 191: 769–776.

91 Amiral J, Bridey F, Dreyfus M et al. Platelet factor 4 complexed to heparin is the target for antibodies generated in heparin-induced thrombocytopenia (letter). *Thromb Haemost* 1992; 68: 95–96.

92 Amiral J, Bridey F, Wolf M et al. Antibodies to macromolecular platelet factor 4 heparin complexes in heparin induced thrombocytopenia: a study of 44 cases. *Thromb Haemost* 1995; 73: 21–28.

93 Amiral J, Marfaing-Koka A, Wolf M et al. Presence of autoantibodies to interleukin-8 or neutrophil-activating peptide-2 in patients with heparin-associated-thrombocytopenia. *Blood* 1996; 88: 410–416.

94 Greinacher A, Poetzsch B, Amiral J, Dummel V, Eichner A, Mueller-Eckhardt C. Heparin-associated thrombocytopenia: isolation of the antibody and characterization of a multimolecular PF4–heparin complex as the major antigen. *Thromb Haemost* 1994; 71: 247–251.

95 Kelton JG, Smith JW, Warkentin TE, Hayward CP, Denomme GA, Horsewood P. Immunoglobulin G from patients with heparin-induced thrombocytopenia binds to a complex of heparin and platelet factor 4. *Blood* 1994; 83: 3232–3239.

96 Visentin GP, Ford SE, Scott JP, Aster RH. Antibodies from

Cambridge Books Online © Cambridge University Press, 2009

patients with heparin-induced thrombocytopenia/thrombosis are specific for platelet factor 4 complexed with heparin or bound to endothelial cells. *J Clin Invest* 1994; 93: 81–88.

97 Gruel Y, Boizard-Boval B, Wautier JL. Further evidence that alpha-granule components such as platelet factor 4 are involved in platelet-IgG-heparin interactions during heparin-associated thrombocytopenia. *Thromb Haemost* 1993; 70: 374–375.

98 Loscalzo J, Melnick B, Handin RI. The interaction of platelet factor four and glycosaminoglycans. *Arch Biochem Biophys* 1985; 240: 446–455.

99 Mayo KH, Ilyina E, Roongta V et al. Heparin binding to platelet factor-4. An NMR and site-directed mutagenesis study: arginine residues are crucial for binding. *Biochem J* 1995; 312: 357–365.

100 Horsewood P, Kelton JG. Investigation of a platelet factor 4 polymorphism on the immune response in patients with heparin-induced thrombocytopenia. *Platelets* 2000; 11: 23–27.

101 Amiral J, Meyer D. Heparin-dependent antigens in heparin-induced antibodies. In Warkentin TE and Greinacher A. eds. I, *Heparin-induced thrombocytopenia*, Marcel Dekker, 2001, 2nd ed.: 137–148.

102 Horsewood P, Warkentin TE, Hayward CP, Kelton JG. The epitope specificity of heparin-induced thrombocytopenia. *Br J Haematol* 1996; 95: 161–167.

103 Ziporen L, Li ZQ, Park KS et al. Defining an antigenic epitope on platelet factor 4 associated with heparin-induced thrombocytopenia. *Blood* 1998; 92: 3250–3259.

104 Suh JS, Aster RH, Visentin GP. Antibodies from patients with heparin-induced thrombocytopenia/thrombosis recognize different epitopes on heparin: platelet factor 4. *Blood* 1998; 91: 916–922.

105 Visentin GP. Heparin-induced thrombocytopenia: molecular pathogenesis. *Thromb Haemost* 1999; 82: 448–456.

106 Visentin GP, Bacsi SG, Aster RH. Molecular immunopathogenesis of heparin-induced thrombocytopenia. In Warkentin TE, Greinacher A. eds. *Heparin-induced thrombocytopenia*, New York: Marcel Dekker; 2001, 2nd ed.: 149–166.

107 Luscher EF, Kaser-Glanzman R. Platelet heparin-neutralizing factor (platelet factor 4). *Thromb Diath Haemorrh* 1975; 33: 66–72.

108 Novotny WF, Maffi T, Mehta RL, Milner PG. Identification of novel heparin-releasable proteins, as well as the cytokines midkine and pleiotrophin, in human postheparin plasma. *Arterioscler Thromb* 1993: 13: 1798–1805.

109 O'Brien JR, Etherington MD, Pashley MA. The heparin-mobilisable pool of platelet factor 4: a comparison of intravenous and subcutaneous heparin and Kabi heparin fragment 2165. *Thromb Haemost* 1985; 54: 735–738.

110 Alban S, Greinacher A. Role of sulfated polysaccharides in the pathogenesis of heparin-induced thrombocytopenia. In Warkentin TE, Greinacher A. eds. *Heparin-induced thrombocytopenia*, New York: Marcel Dekker; 2001, 2nd ed.: 167–188.

111 Greinacher A, Alban S, Dummel V, Franz G, Mueller-Eckhardt C. Characterization of the structural requirements for a carbohydrate based anticoagulant with a reduced risk of inducing the immunological type of heparin-associated thrombocytopenia. *Thromb Haemost* 1995; 74: 886–892.

112 Maccarana M, Lindahl U. Mode of interaction between platelet factor 4 and heparin. *Glycobiology* 1993; 3: 271–277.

113 Handin RI, Cohen HJ. Purification and binding properties of human platelet factor four. *J Biol Chem* 1976; 251: 4273–4282.

114 Petitou M, Duchaussoy P, Jaurand G et al. Synthesis and pharmacological properties of a close analogue of an antithrombotic pentasaccharide (SR 90107A/ORG 31540). *J Med Chem* 1997; 40: 1600–1607.

115 Chong BH, Magnani HN. Danaparoid for the treatment of heparin-induced thrombocytopenia. In Warkentin TE, Greinacher A. eds. *Heparin-induced thrombocytopenia*, Marcel Dekker; 2001, 2nd ed.: 323–348.

116 Petitou M, Herault JP, Bernat A et al. Synthesis of thrombin-inhibiting heparin mimetics without side effects. *Nature* 1999; 398: 417–422.

117 Horne III MK, Alkins BR. Platelets binding of IgG from patients with heparin-induced thrombocytopenia. *J Lab Clin Med* 1996; 127: 435–442.

118 Dawes J, Pumphrey CW, McLaren KM, Prowse CV, Pepper DS. The in vivo release of human platelet factor 4 by heparin. *Thromb Res* 1982; 27: 65–76.

119 Lee DH, Warkentin TE. Frequency of heparin-induced thrombocytopenia. in: Warkentin TE and Greinacher A. eds. *Heparin-induced thrombocytopenia*, Marcel Dekker; 2001, 2nd ed.: 87–122.

120 Arepally G, McKenzie SE, Jiang X-M, Poncz M, Cines DB. FcγRIIA H/R131 polymorphism, subclass-specific IgG anti-heparin/platelet factor 4 antibodies and clinical course in patients with heparin-induced thrombocytopenia and thrombosis. *Blood* 1997; 89: 370–375.

121 Denomme GA, Warkentin TE, Horsewood P, Sheppard J-A I, Warner MN, Kelton JG. Activation of platelets by sera containing IgG1 heparin-dependent antibodies: an explanation for the predominance of the FcγRIIa 'low responder' (his_{131}) gene in patients with heparin-induced thrombocytopenia. *J Lab Clin Med* 1997; 130: 278–284.

122 Suh JS, Malik MI, Aster RH, Visentin GP. Characterization of the humoral immune response in heparin-induced thrombocytopenia. *Am J Hematol* 1997; 54: 196–201.

123 Amiral J, Wolf M, Fischer A, Boyer-Neumann C, Vissac A, Meyer D. Pathogenicity of IgA and/or IgM antibodies to heparin-PF4 complexes in patients with heparin-induced thrombocytopenia. *Br J Haematol* 1996; 92: 954–959.

124 Sylvester L, Yoshimura T, Sticherling M et al. Neutrophil attractant Protein-1-immunoglobulin G immune complexes and free anti-NAP-1 antibody in normal human serum. *J Clin Invest* 1992; 90: 471–481.

125 Reitamo S, Remitz A, Varga J et al. Demonstration of interleukin 8 and autoantibodies to interleukin 8 in the serum of patients with sytemic sclerosis and related disorders. *Arch Dermatol* 1993; 129: 189–193.

Cambridge Books Online © Cambridge University Press, 2009

126 Bendtzen K, Hansen MB, Ross C, Poulsen LK, Svenson M. Cytokines and autoantibodies to cytokines. *Stem Cells* 1995; 13: 206–222.

127 Denomme GA. The platelet Fc receptor in heparin-induced thrombocytopenia. In: Warkentin TE and Greinacher A. eds. *Heparin-induced thrombocytopenia*, Marcel Dekker, 2001, 2nd ed.: 189–214.

128 Kelton JG, Sheridan D, Santos A et al. *Heparin-induced thrombocytopenia*: Laboratory studies. *Blood* 1988; 72: 925–930.

129 Chong BH, Pilgrim RL, Cooley MA, Chesterman CN. Increased expression of platelet IgG Fc receptors in immune heparin-induced thrombocytopenia. *Blood* 1993; 81: 988–993.

130 Rappaport EF, Cassel DL, Walterhouse DO et al. A soluble form of the human Fc receptor FcγRIIa: cloning, transcript analysis and detection. *Exp Hematol* 1993; 21: 689–696.

131 Gachet C, Astier A, de la Salle H et al. Release of FcγRIIa2 by activated platelets and inhibition of anti-CD9-mediated platelet aggregation by recombinant FcγRIIa2. *Blood* 1995; 85: 698–704.

132 Saffroy R, Bachelot-Loza C, Fridman WH, Aiach M, Teullaud J-L, Rendu F. Plasma levels of soluble Fc receptors II (sCD32) and III (sCD16) in patients with heparin-induced thrombocytopenia. *Thromb Haemost* 1997; 78: 970–971.

133 Warmerdam PAM, van de Winkel JGJ, Gosselin EJ, Capel PJA. Molecular basis for a polymorphism of human Fcγ receptor II (CD32w). *J Exp Med* 1990; 172: 19–25.

134 Horsewood P, Hayward CPM, Warkentin TE, Kelton JG. Investigation of the mechanisms of monoclonal antibody-induced platelet activation. *Blood* 1991; 78: 1019–1026.

135 Bachelot C, Saffroy R, Gandrille S, Aiach M, Rendu F. Role of FcγRIIA gene polymorphism in human platelet activation by monoclonal antibodies. *Thromb Haemost* 1995; 74: 1557–1563.

136 Bachelot-Loza C, Saffroy R, Lasne D, Chatellier G, Aiach M, Rendu F. Importance of the FcRIIA-Arg/His-131 polymorphism in heparin-induced thrombocytopenia diagnosis. *Thromb Haemost* 1998; 79: 523–528.

137 Burgess JK, Lindeman R, Chesterman CN, Chong BH. Single amino acid mutation of Fc receptor is associated with the development of heparin-induced thrombocytopenia. *Br J Haematol* 1995; 91: 761–766.

138 Brandt J, Isenhart CE, Osborne JM, Ahmed A, Anderson CL. On the role of platelet Fc gamma RIIa phenotype in heparin-induced thrombocytopenia. *Thromb Haemost* 1995; 74: 1564–1572.

139 Carlsson LE, Santoso S, Baurichter G et al. *Heparin-induced thrombocytopenia*: new insights into the impact of the FcγRIIa-R-H131 polymorphism. *Blood* 1998; 92: 1526–1531.

140 Polgar J, Eichler P, Greinacher A, Clemetson KJ. Adenosine diphosphate (ADP) and ADP receptor play a major role in platelet activation/aggregation induced by sera from heparin-induced thrombocytopenia patients. *Blood* 1998; 91: 549–554.

141 Cines DB, Tomaski A, Tannenbaum S. Immune endothelial-cell injury in heparin-associated thrombocytopenia. *N Engl J Med* 1987; 316: 581–589.

142 Herbert JM, Savi P, Jeske WP, Walenga JM. Effect of SR 121566A, a potent GP IIb–IIIa antagonist, on the HIT serum/heparin-induced platelet mediated activation of human endothelial cells. *Thromb Haemost* 1998; 80: 326–331.

143 Warkentin TE, Elavathil LJ, Hayward CPM, Johnston MA, Russett JI, Kelton JG. The pathogenesis of venous limb gangrene associated with heparin-induced thrombocytopenia. *Ann Intern Med* 1997; 127: 804–812.

144 Greinacher A, Eichler P, Lubenow N, Kwasny H, Luz M. Heparin-induced thrombocytopenia with thromboembolic complications: meta-analysis of two prospective trials to assess the value of parenteral treatment with lepirudin and its therapeutic aPTT range. *Blood* 2000; 96: 846–851.

145 Chong BH, Murray B, Berndt MC, Dunlop LC, Brighton T, Chesterman CN. Plasma P-selectin is increased in thrombotic consumptive platelet disorders. *Blood* 1994; 83: 1535–1541.

146 Warkentin TE, Hayward CP, Boshkov LK et al. Sera from patients with heparin-induced thrombocytopenia generate platelet-derived microparticles with procoagulant activity: An explanation for the thrombotic complications of heparin-induced thrombocytopenia. *Blood* 1994; 84: 3691–3699.

147 Pouplard C, Iochmann S, Renard B et al. Induction of monocyte tissue factor expression by antibodies to platelet factor 4 developed in heparin-induced thrombocytopenia. *Blood* 2000; 96: 530a (abstract).

148 Lee DH, Warkentin TE, Denomme GA, Lagrotteria DD, Kelton JG. Factor V Leiden and thrombotic complications in heparin-induced thrombocytopenia. *Thromb Haemost* 1998; 79: 50–53.

149 Carlsson LE, Papenberg S, Eichler P, Schröder W, Herrmann F, Greinacher A. Factor V Leiden, prothrombin 20210, MTHFR polymorphisms and the development of thrombotic complications in heparin-induced thrombocytopenia (HIT) (abstract). *Ann Hematol* 2000; 79(Suppl 1): A92.

150 Warkentin TE, Levine MN, Hirsh J et al. Heparin-induced thrombocytopenia in patients treated with low-molecular-weight heparin or unfractionated heparin. *N Engl J Med* 1995; 332: 1330–1335.

151 Warkentin TE, Levine MN, Hirsh J, Klama LN, Kelton JG. Formation of heparin-induced thrombocytopenia IgG without thrombocytopenia: analysis of a clinical trial (abstr). *Blood* 1995; 86(suppl 1): 537.

152 Warkentin TE, Ling E, Ho A, Sheppard JI. 'Incidental' unfractionated heparin (UFH) vs normal saline (NS) flushes for intraoperative invasive catheters and the frequency of formation of heparin-induced thrombocytopenia IgG antibodies (HIT-IgG): a randomized, controlled trial (abstr). *Blood* 1998; 92(suppl 1): 91b.

153 Warkentin TE, Simpson PJ, Sheppard JI, Moore JC, Horsewood P, Kelton JG. Importance of patient population in the frequency of HIT: a comparison of activation and

Cambridge Books Online © Cambridge University Press, 2009

antigen assays (abstr). *Thromb Haemost* 1999; 82(suppl): 363–364.
154 Amiral J, Peynaud-Debayle E, Wolf M, Bridey F, Vissac A-M, Meyer D. Generation of antibodies to heparin-PF4 complexes without thrombocytopenia in patients treated with unfractionated or low-molecular-weight heparin. *Am J Hematol* 1996; 52: 90–95.
155 Visentin GP, Malik M, Cyganiak KA, Aster RH. Patients treated with unfractionated heparin during open heart surgery are at high risk to form antibodies reactive with heparin: platelet factor 4 complexes. *J Lab Clin Med* 1996; 128: 376–383.
156 Kappers-Klunne MC, Boon DMS, Hop WCJ et al. Heparin-induced thrombocytopenia and thrombosis: a prospective analysis of the incidence in patients with heart and cerebrovascular diseases. *Br J Haematol* 1997; 96: 442–446.
157 Bauer TL, Arepally G, Konkle BA et al. Prevalence of heparin-associated antibodies without thrombosis in patients undergoing cardiopulmonary bypass surgery. *Circulation* 1997; 95: 1242–1246.
158 Trossaert M, Gaillard A, Cyganiak KA, Aster RH. High incidence of anti-heparin/platelet factor 4 antibodies after cardiopulmonary bypass. *Br J Haematol* 1998; 101: 653–655.
159 Pouplard C, May MA, Iochmann S et al. Antibodies to platelet factor 4-heparin after cardiopulmonary bypass in patients anticoagulated with unfractionated heparin or a low-molecular-weight heparin: clinical implications for heparin-induced thrombocytopenia. *Circulation* 1999; 99: 2530–2536.
160 Ganzer D, Gutezeit A, Mayer G. [Gefahrenpotentiale in der medikamentösen Thromboseprophylaxe – niedermolekulares Heparin versus Standardheparin]. *Z Orthop Ihre Grenzgeb* 1999; 137: 457–461.
161 Warkentin TE, Kelton JG. Temporal aspects of heparin-induced thrombocytopenia. *N Engl J Med* 2001; 344: 1286–1292.
162 Kempf R, Eichner A, Lubenow N, Ranze O, Greinacher A. Heparin-induced thrombocytopenia: temporal pattern of platelet counts in patients being firstly exposed or reexposed to heparin. *Ann Hematol* 2000; 79 (suppl 1): A92.
163 WarkentinTE, Kelton JE. A 14-year study of heparin-induced thrombocytopenia. *Am J Med* 1996; 101: 502–507.
164 Ernest D, Fisher MM. Heparin-induced thrombocytopaenia complicated by bilateral adrenal hemorrhage. *Intens Care Med* 1991; 17: 238–240.
165 Meyer-Lindenberg A, Quenzel EM, Bierhoff E, Wolff H, Schindler E, Biniek R. Fatal cerebral venous sinus thrombosis in heparin-induced thrombotic thrombocytopenia. *Eur Neurol* 1997; 37: 191–192.
166 Pohl C, Klockgether T, Greinacher A, Hanfland P, Harbrecht U. Neurological complications in heparin-induced thrombocytopenia. *Lancet* 1999; 353: 1678–1679.
167 Warkentin TE. Heparin-induced skin lesions. *Br J Haematol* 1996; 92: 494–497.
168 Warkentin TE, Sikov WM, Lillicrap DP. Multicentric warfarin-induced skin necrosis complicating heparin-induced thrombocytopenia. *Am J Hematol* 1999; 62: 44–48.
169 Warkentin TE, Soutar RL, Panju A, Ginsberg JS. Acute systemic reactions to intravenous bolus heparin therapy: characterization and relationship to heparin-induced thrombocytopenia (abstr). *Blood* 1992; 80(Suppl 1): 160a.
170 Popov D, Zarrabi MH, Foda H, Graber M. Pseudopulmonary embolism: acute respiratory distress in the syndrome of heparin-induced thrombocytopenia. *Am J Kidney Dis* 1997; 29: 449–452.
171 Tholl U, Greinacher A, Overdick K, Anlauf M. Life-threatening anaphylactic reaction following parathyroidectomy in a dialysis patient with heparin-induced thrombocytopenia. *Nephrol Dial Transpl* 1997; 12: 2750–2755.
172 Lubenow N, Eichler P, Albrecht D et al. Very low platelet counts in post-transfusion purpura falsely diagnosed as heparin-induced thrombocytopenia. Report of four cases and review of literature. *Thromb Res* 2000; 100: 115–125.
173 Warkentin TE. Pseudo-heparin-induced thrombocytopenia: In Warkentin TE, Greinacher A. eds. *Heparin-induced thrombocytopenia*, Marcel Dekker; 2001, 2nd ed.: 271–290.
174 Arnout J. The pathogenesis of the antiphospholipid syndrome: a hypothesis based on parallelisms with heparin-induced thrombocytopenia. *Thromb Haemost* 1996; 75: 536–541.
175 Warkentin TE, Greinacher A. Laboratory testing for heparin-induced thrombocytopenia. In Warkentin TE, Greinacher A. eds. *Heparin-induced thrombocytopenia*, New York: Marcel Dekker; 2001, 2nd ed.: 231–270.
176 Sheridan D, Carter C, Kelton JG. A diagnostic test for heparin-induced thrombocytopenia. *Blood* 1986; 67: 27–30.
177 Greinacher A, Michels I, Kiefel V, Mueller-Eckhardt C. A rapid and sensitive test for diagnosing heparin-associated thrombocytopenia. *Thromb Haemost* 1991; 66: 734–736.
178 Eichler P, Budde U, Haas S et al. First workshop for detection of heparin-induced antibodies: validation of the heparin-induced platelet-activation test (HIPA) in comparison with a PF4/heparin ELISA. *Thromb Haemost* 1999; 81: 625–629.
179 Stewart MW, Etches WS, Boshkov LK, Gordon PA. Heparin-induced thrombocytopenia: an improved method of detection based on lumi-aggregometry. *Br J Haematol* 1995; 91: 173–177.
180 Lee DH, Warkentin TE, Denomme GA, Hayward CPM, Kelton JG. A diagnostic test for heparin-induced thrombocytopenia: detection of platelet microparticles using flow cytometry. *Br J Haematol* 1996; 95: 724–731.
181 Tomer A. A sensitive and specific functional flow cytometric assay for the diagnosis of heparin-induced thrombocytopenia. *Br J Haematol* 1997; 98: 648–656.
182 Kelton JG, Sheridan D, Brain H, Powers PJ, Turpie AG, Carter CJ. Clinical usefulness of testing for a heparin-dependent platelet-aggregating factor in patients with suspected heparin-associated thrombocytopenia. *J Lab Clin Med* 1984; 103: 606–612.
183 Pfueller SL, David R. Different platelet specificities of heparin-dependent platelet aggregating factors in heparin-associated immune thrombocytopenia. *Br J Haematol* 1986; 64: 149–159.
184 Favaloro EJ, Bernal-Hoyos E, Exner T, Koutts J. Heparin-

Cambridge Books Online © Cambridge University Press, 2009

induced thrombocytopenia: laboratory investigation and confirmation of diagnosis. *Pathology* 1992; 24: 177–183.

185 Greinacher A, Amiral J, Dummel V, Vissac A, Keifel V, Mueller-Eckhardt C. Laboratory diagnosis of heparin-associated thrombocytopenia and comparison of platelet aggregation test, heparin-induced platelet activation test, and platelet factor 4/heparin enzyme-linked immunosorbent assay. *Transfusion* 1994; 34: 381–385.

186 Visentin GP, Moghaddam M, Collins JL, McFarland JG, Aster RH. Antibodies associated with heparin-induced thrombocytopenia (HIT) report conformational changes in platelet factor 4 (PF4) induced by linear polyanionic compounds (Abst.). *Blood* 1997; 90 (suppl 1): 460a.

187 Newman PM, Swanson RL, Chong BH. IgG Binding to PF4-heparin complexes in the fluid phase and cross-reactivity with low molecular weight heparin and heparinoid. *Thromb Haemost* 1998; 80: 292–297.

188 Ngai PK, Ackermann F, Wendt H, Savoca R, Bosshard HR. Protein A antibody-capture ELISA (PACE): an ELISA format to avoid denaturation of surface-absorbed antigens. *J Immunol Meth* 1993; 158: 267–276.

189 Meyer O, Salama A, Pittet N, Schwind P. Rapid detection of heparin-induced platelet antibodies with particle gel immunoassay (ID-HPF4). *Lancet* 1999; 354: 1525–1526.

190 Warkentin TE. Limitations of conventional treatment options for heparin-induced thrombocytopenia. *Semin Hematol* 1998; 35: 17–25.

191 Boon DMS, Michiels JJ, Stibbe J, van Vliet HHDM, Kappers-Klunne MC. Heparin-induced thrombocytopenia and antithrombotic therapy (letter). *Lancet* 1994; 344: 1296.

192 Greinacher A, Völpel H, Janssens U et al. Recombinant hirudin (lepirudin) provides safe and effective anticoagulation in patients with the immunologic type of heparin-induced thrombocytopenia: a prospective study. *Circulation* 1999; 99: 73–80.

193 Greinacher A, Janssens U, Berg G et al. Lepirudin (recombinant hirudin) for parenteral anticoagulation in patients with heparin-induced thrombocytopenia. *Circulation* 1999; 100: 587–593.

194 Greinacher A. Treatment of heparin-induced thrombocytopenia. *Thromb Haemost* 1999; 82: 457–467.

195 Greinacher A, Warkentin TE. Treatment of heparin-induced thrombocytopenia: an overview. In Warkentin TE and Greinacher A. eds. *Heparin-induced thrombocytopenia*, Marcel Dekker; 2001, 2nd ed.: 291–322.

196 Weitz JI, Hudoba M, Massel D, Maraganore J, Hirsh J. Clot-bound thrombin is protected from inhibition by heparin-antithrombin III but is susceptible to inactivation by antithrombin III-independent inhibitors. *J Clin Invest* 1990; 86: 385–391.

197 Weitz JI, Leslie B, Hudoba M. Thrombin binds to soluble fibrin degradation products where it is protected from inhibition by heparin-antithrombin but susceptible to inactivation by antithrombin-independent inhibitors. *Circulation* 1998; 97: 544–552.

198 Hogg PJ, Jackson CM, Labanowski JK, Bock PE. Binding of fibrin monomer and heparin to thrombin in a ternary complex alters the environment of the thrombin catalytic site, reduces affinity for hirudin, and inhibits cleavage of fibrinogen. *J Biol Chem* 1996; 271: 26088–26095.

199 Hogg PJ, Bock PE. Modulation of thrombin and heparin activities by fibrin. *Thromb Haemost* 1997; 77: 424–433.

200 Lubenow N, Greinacher A. Heparin-induced thrombocytopenia. Recommendations for optimal use of recombinant hirudin. *BioDrugs* 2000; 14: 109–125.

201 Schiele F, Vuillemenot A, Kramarz P et al. A pilot study of subcutaneous recombinant hirudin (HBW 023) in the treatment of deep vein thrombosis. *Thromb Haemost* 1994; 71: 558–562.

202 Eriksson BI, Ekman S, Lindbratt S et al. Prevention of thromboembolism with use of recombinant hirudin. Results of a double-blind, multicenter trial comparing the efficacy of desirudin (Revasc) with that of unfractionated heparin in patients having a total hip replacement. *J Bone Joint Surg Am* 1997; 79: 326–333.

203 Eriksson BI, Wille-Jörgensen P, Kälebo P et al. A comparison of recombinant hirudin with a low-molecular weight heparin to prevent thromboembolic complications after total hip replacement. *N Engl J Med* 1997; 337: 1329–3135.

204 Markwardt F. Development of hirudin as an antithrombotic agent. *Semin Thromb Haemost* 1989; 15: 269–282.

205 Nowak G, Bucha E, Gööck T, Prasa D, Thieler H. Pharmakokinetik von Hirudin bei gestörter Nierenfunktion. *Hämostaseologie* 1991; 11: 152–157.

206 Nowak G, Bucha E, Brauns I, Czerwinski R. Anticoagulation with r-hirudin in regular haemodialysis with heparin-induced thrombocytopenia (HIT II). The first long term application of r-hirudin in a haemodialysis patient. *Wien Klin Wochenschr* 1997; 109: 354–358.

207 Vanholder R, Camez A, Veys NM et al. Recombinant Hirudin: A specific thrombin-inhibiting anticoagulant for hemodialysis. *Kidney Int* 1994; 45: 1754–1759.

208 Vanholder R, Camez A, Veys N, Van Loo A, Dhondt A, Ringoir S. Pharmacokinetics of recombinant hirudin in hemodialyzed end-stage renal failure patients. *Thromb Haemost* 1997; 77: 650–655.

209 Nowak G, Bucha E, Gööck T, Thieler M, Markwardt F. Pharmacology of r-hirudin in renal impairment. *Thromb Res* 1992; 66: 707–715.

210 Verstraete M, Nurmohamed M, Kienast J et al. European Hirudin in Thrombosis Group. Biologic effects of recombinant hirudin (CGP 39393) in human volunteers. *J Am Coll Cardiol* 1993; 22: 1080–1088.

211 Marbet GA, Verstraete M, Kienast J et al. Clinical pharmacology of intravenously administered recombinant desulfatohirudin (CGP 39393) in healthy volunteers. *J Cardiovasc Pharmacol* 1993; 22: 364–372.

212 Bridey F, Dreyfus M, Parent F et al. Recombinant hirudin (HBW 023): biological data of ten patients with severe venous thrombo-embolism. *Am J Hematol* 1995; 49: 67–72.

213 Zeymer U, von Essen R, Tebbe U et al. Recombinant hirudin

Cambridge Books Online © Cambridge University Press, 2009

and front-loaded alteplase in acute myocardial infarction: Final results of a pilot study. HIT-I (hirudin for the improvement of thrombolysis). *Eur Heart J* 1995; 16(suppl D): 22–27.

214 Zeymer U, von Essen R, Tebbe U et al. ALKK Study Group. Frequency of 'optimal anticoagulation' for acute myocardial infarction after thrombolysis with front-loaded recombinant tissue-type plasminogen activator and conjunctive therapy with recombinant hirudin (HBW 023). *Am J Cardiol* 1995; 76: 997–1001.

215 Hafner G, Rupprecht HJ, Luz M et al. Recombinant hirudin as a periprocedural antithrombotic in coronary angioplasty for unstable angina pectoris [see comments]. *Eur Heart J* 1996; 17: 1207–1215.

216 Nurmohamed MT, Berckmans RJ, Morrien-Salomons WM et al. Monitoring anticoagulant therapy by activated partial thromboplastin time: hirudin assessment. *Thromb Haemost* 1994; 72: 685–692.

217 Pötzsch B, Hund S, Madlener K, Unkrig C, Muller-Berghaus G. Monitoring of recombinant hirudin: assessment of a plasma-based ecarin clotting time assay. *Thromb Res* 1997; 86: 373–383.

218 Pötzsch B, Madlener K, Seelig C, Riess C, Greinacher A, Müller-Berghaus G. Monitoring of r-hirudin anticoagulation during cardiopulmonary bypass – Assessment of the whole blood ecarin clotting time. *Thromb Haemost* 1997; 77: 920–925.

219 Kornalik F, Blombäck B. Prothrombin activation induced by ecarin – a prothrombin converting enzyme from *Echis carinatus* venom. *Thromb Res* 1975; 6: 57–63.

220 Morita T, Iwanaga S, Suzuki T. The mechanism of activation of bovine prothrombin by an activator isolated from *Echis carinatus* venom and characterization of the new active intermediates. *J Biochem* 1976; 79: 1089–1108.

221 Novoa E, Seegers WH. Mechanisms of alpha-thrombin and beta-thrombin-E formation: use of ecarin for isolation of meizothrombin 1. *Thromb Res* 1980; 18: 657–668.

222 Nishida S, Fujita T, Kohno N et al. cDNA cloning and deduced amino acid sequence of prothrombin activator (ecarin) from Kenyan *Echis carinatus* venom. *Biochemistry* 1995; 34: 1771–1778.

223 Pötzsch B, Madlener K. Management of cardiopulmonary bypass anticoagulation in patients with heparin-induced thrombocytopenia. In Warkentin TE and Greinacher A. eds. *Heparin-induced thrombocytopenia*, Marcel Dekker; 2001, 2nd ed.: 429–444.

224 Schiele F, Vuillemenot A, Kramarz P et al. Use of recombinant hirudin as antithrombotic treatment in patients with heparin-induced thrombocytopenia. *Am J Hematol* 1995; 50: 20–25.

225 Schiffmann H, Unterhalt M, Harms K, Figulla HR, Völpel H, Greinacher A. Successful treatment of heparin-induced thrombocytopenia (HIT) type II in childhood with recombinant hirudin. *Monatsschr Kinderheilkd* 1997; 145: 606–612.

226 Harenberg J, Huhle G, Piazolo L, Wang LU, Heene DL. Anticoagulation in patients with heparin-induced thrombocytopenia type II. *Sem Thromb Haemost* 1997; 23: 189–196.

227 Schmidt OH, Lang W. Heparin-induced thrombocytopenia with thromboembolic arterial occlusion treated with recombinant hirudin. *N Engl J Med* 1997; 337: 1389.

228 Wagner AD, Kleber G, Lindner A, Pötzsch B, Fleig WE. 19jähriger Patient mit generalisiertem Krampfanfall und rezidivierenden Thrombosen unter Heparintherapie. *Internist* 1998; 39: 766–769.

229 Olbrich K, Wiersbitzky M, Wacke W et al. Atypical heparin-induced thrombocytopenia complicated by intracardiac thrombus, effectively treated with ultra-low-dose rt-PA lysis and recombinant hirudin (Lepirudin). *Blood Coagul Fibrinol* 1998; 9: 273–277.

230 Janssens U, Breithardt OA, Greinacher A. Successful thrombolysis of right atrial and ventricle thrombi encircling a temporary pacemaker lead in a patient with heparin-induced thrombocytopenia type II. *Pacing Clin Electrophysiol* 1999; 22: 678–681.

231 Madei W, Klieser HP, Hoerauf K. Heparin-induced thrombocytopenia as a cause of persistent bleeding after cardiac pacemaker implantation (German). *Dtsch Med Wochenschr* 1999; 124: 487–490.

232 Eichler P, Friesen HJ, Lubenow N, Jaeger B, Greinacher A. Antihirudin antibodies in patients with heparin-induced thrombocytopenia treated with lepirudin: incidence, effects on aPTT, and clinical relevance. *Blood* 2000; 96: 2373–2378.

233 Huhle G, Song X, Wang LC, Hoffmann U, Harenberg J. Generation and disappearance of antihirudin antibodies during treatment with r-hirudin. *Fibrinol Proteol* 1998; 12 (suppl 2): 91–113.

234 Huhle G, Hoffmann U, Song XH, Heene DL, Harenberg J. Immunantwort auf rekombinantes Hirudin: Eine Übersicht (Immune response to recombinant hirudin: an overview). *Haemostaseologie* 1999; 19: 50–54.

235 Huhle, G, Hoffmann U, Song X, Wang LC, Heene DL, Harenberg J. Immunologic response to recombinant hirudin in HIT type II patients during long-term treatment. *Br J Haematol* 1999; 106: 195–201.

236 Hursting MJ, Alford KL, Becker JC et al. Novastan (brand of argatroban): a small-molecule, direct thrombin inhibitor. *Semin Thromb Haemost* 1997; 23: 503–516.

237 Lewis BE, Wallis DE, Berkowitz SD, Matthai WH for the ARG-911 Study Investigators. Argatroban anticoagulant therapy in patients with heparin-induced thrombocytopenia. *Circulation* 2001; 103: 1038–1043.

238 Magnani HN. Heparin-induced thrombocytopenia (HIT): an overview of 230 patients treated with Orgaran (Org 10172). *Thromb Haemost* 1993; 70: 554–561.

239 Magnani HN. Orgaran (danaparoid sodium) use in the syndrome of heparin-induced-thrombocytopenia. Proceedings of a workshop held in London 1996, Nov 1. *Platelets* 1997; 8: 74–81.

240 Chong AS, Gallus JF, Cade H et al. Prospective randomised open-label comparison of danaparoid with dextran 70 in the

Cambridge Books Online © Cambridge University Press, 2009

treatment of heparin-induced thrombocytopenia with thrombosis. *Thromb Haemost* 2001; 86: 1170–1175.

241 Meulemann DG. Orgaran (Org 10172): Its pharmacological profile in experimental models. *Haemostasis* 1992; 22: 58–65.

242 Skoutakis VA. Danaparoid in the prevention of thromboembolic complications. *Ann Pharmacother* 1997; 31: 876–887.

243 Laposata M, Green D, Van Cott EM, Barrowcliffe TW, Goodnight SH, Sosolik RC. College of American Pathologists Conference Therapy. The clinical use and laboratory monitoring of low-molecular-weight heparin, danaparoid, hirudin and related compounds, and argatroban. *Arch Pathol Lab Med* 1998; 122: 799–807.

244 Insler SR, Kraenzler EJ, Barholomew JR, Kottke-Merchant K, Lytle B, Starr NJ. Thrombosis during the use of the heparinoid Organon 10172 in a patient with heparin-induced thrombocytopenia. *Anesthesiology* 1997; 86: 495–498.

245 Warkentin TE. Danaparoid (Orgaran) for the treatment of heparin-induced thrombocytopenia (HIT) and thrombosis: effects on in vivo thrombin and cross-linked fibrin generation, and evaluation of the clinical significance of in vitro cross-reactivity of danaparoid for HIT-IgG. *Blood* 1996; 88: 626a.

246 Baumgartel MW, Eichler P, Glockner WM, Ranze O, Greinacher A. Heparin-induced thrombocytopenia (HIT): in vitro and in vivo cross-reactivity to danaparoid sodium and successful treatment with recombinant hirudin. *Eur J Haematol* 2000; 65: 148–149.

247 van Wyk V, Badenhorst PN, Luus HG, Kotzé HF. A comparison between the use of recombinant hirudin and heparin during hemodialysis. *Kidney Int* 1995; 48: 1338–1343.

248 Fischer KG. Hemodialysis in heparin-induced thrombocytopenia. In Warkentin TE and Greinacher A. eds. *Heparin-induced thrombocytopenia*, Marcel Dekker; 2001, 2nd ed.: 409–428.

249 Hempel S, Lubenow N, Greinacher A. Nierenersatztherapie unter r-hirudin (Refludan) bei Heparin-induzierter Thrombozytopenie Typ II (Abstract). *Infusionsther Transfusionsmed* 1998; 25: 57.

250 Matsuo T, Koide M, Kario K. Application of argatroban, direct thrombin inhibitor, in heparin-intolerant patients requiring extracorporeal circulation. *Artif Organs* 1997; 21: 1035–1038.

251 Ireland H, Lane DA, Flynn A, Anastassiades E, Curtis JR. The anticoagulant effect of heparinoid Org 10172 during haemodialysis: an objective assessment. *Thromb Haemost* 1986; 55: 271–275.

252 Rowlings PA, Mansberg R, Rozenberg MC, Evans S, Murray B. The use of a low-molecular weight heparinoid (Org 10172) for extracorporeal procedures in patients with heparin-dependent thrombocytopenia and thrombosis. *Aust NZ J Med* 1991; 21: 52–54.

253 Thannheimer A, Preuner JG, Pötzsch B, Birnbaum DE, Völpel A, Greinacher A. Heparin-associated thrombocytopenia in open-heart surgery: heparin or hirudin as anticoagulant: The antibody decides. *Ann Hematol* 1996; 72(Suppl I): P222.

254 Riess FC, Bleese N, Kormann et al. Recombinant hirudin is a heparin alternative in cardiac surgery (letter). *Cardiothorac Vasc Anesth* 1997; 11: 538–539.

255 Koster A, Kuppe H, Hetzer R, Sodian R, Crystal GJ, Mertzlufft F. Emergent cardiopulmonary bypass in five patients with heparin-induced thrombocytopenia type II employing recombinant hirudin. *Anesthesiology* 1998; 89: 777–780.

256 Riess FC, Löwer C, Seelig C et al. Recombinant hirudin as a new anticoagulant during cardiac operations instead of heparin: successful for aortic valve replacement in man. *J Thorac Cardiovasc Surg* 1995; 110: 265–267.

257 Riess FC, Pötzsch B, Bader R et al. A case report on the use of recombinant hirudin as an anticoagulant for cardiopulmonary bypass in open heart surgery. *Eur J Cardiothoracic Surg* 1996; 10: 386–368.

258 Riess FC, Poetzsch B, Mueller-Berghaus G. Recombinant hirudin as an anticoagulant during cardiac surgery. In: Pifarré R, ed. *New anticoagulants for the cardiovascular patient*. Philadelphia: Hanley & Belfus, Inc; 1997: 197–222.

259 Magnani HN, Beijering RJR, ten Cate JW, Chong BH. Orgaran anticoagulation for cardiopulmonary bypass in patients with heparin-induced thrombocytopenia. In Pifarré, R, ed. *New anticoagulants for the cardiovascular patient*. Philadelphia: Hanley & Belfus 1997.

260 Olinger GN, Hussey CV, Olive JA, Malik MI. Cardiopulmonary bypass for patients with previously documented heparin-induced platelet aggregation. *J Thorac Cardiovasc Surg* 1984; 87: 673–677.

261 Pötzsch B, Klovekorn WP, Madlener K. Use of heparin during cardiopulmonary bypass in patients with a history of heparin-induced thrombocytopenia. *N Engl J Med* 2000; 343: 515.

262 Ranze O, Ranze P, Magnani HN, Greinacher A. Heparin-induced thrombocytopenia in paediatric patients – a review of the literature and a new case treated with danaparoid sodium. *Eur J Pediatr* 1999; 158 (Suppl3): S130–S133.

263 Akzo-Organon, Oss, Netherlands, *Danaparoid Product Information*, 1999.

264 Markwardt F, Fink G, Kaiser B et al. Pharmacological survey of recombinant hirudin. *Pharmazie* 1988; 43: 202–207.

265 Huhle G, Geberth M, Hoffmann U, Heene DL, Harenberg J. Management of heparin-associated thrombocytopenia in pregnancy with subcutaneous r-hirudin. *Gynecol Obstet Invest* 2000; 49: 67–69.

266 Lindhoff-Last E, Willeke A, Thalhammer C, Nowak G, Bauersachs R. Hirudin treatment in a breastfeeding woman. *Lancet* 2000; 355: 467–468.

267 Greinacher A, Eckhardt T, Mussmann J, Mueller-Eckhardt C. Pregnancy complicated by heparin associated thrombocytopenia: management by a prospectively in vitro selected heparinoid (Org 10172). *Thromb Res* 1993; 71: 123–126.

268 Selleng S, Lubenow N, Wollert H-G, Müllejans B, Greinacher A. Emergency cardiopulmonary bypass in a bilaterally nephrectomized patient with a history of heparin-induced thrombocytopenia: successful reexposure to heparin. *Ann Thorac Surg* 2001, 71: 1041–1042.

Cambridge Books Online © Cambridge University Press, 2009

40

Thrombotic thrombocytopenic purpura and hemolytic uremic syndrome

Miha Furlan

Central Hematology Laboratory, University Hospital, Inselspital, Bern, Switzerland

Introduction

Thrombotic thrombocytopenic purpura (TTP) is a disseminated form of thrombotic microangiopathy that was initially described by Moschowitz in 1924 as a new disease[1]. He reported an abrupt onset of petechiae, anemia and microscopic hematuria in a 16-year-old girl, who presented with fever and malaise and died 13 days later. He observed widespread hyaline microthrombi in the terminal arterioles and capillaries and interpreted them as agglutinated and hyalinized erythrocytes caused by 'a powerful poison which had both agglutinative and hemolytic properties'[2]. A congenital form of TTP was reported[3] by Schulman et al., who suggested that the pathogenesis was due to deficiency of a platelet-stimulating factor in the patient's plasma. A similar congenital deficiency of a plasma factor, important in platelet and red-cell survival, was later described by Schulman in another patient with chronic relapsing TTP[4]. Familial predisposition to TTP was implicated from observations of siblings suffering from recurrent TTP[5,6]. The congenital form of TTP has been occasionally described in the literature as Upshaw–Schulman syndrome.

TTP is characterized by the pentad of severe thrombocytopenia, microangiopathic hemolysis with erythrocyte fragmentation, neurologic deficit, renal dysfunction, and fever[7,8]. A TTP-like disorder, the hemolytic uremic syndrome (HUS), usually occurs in children, and is characterized by the triad of thrombocytopenia, microangiopathic hemolytic anemia and renal dysfunction[9]. The diagnosis of TTP is usually made in adult patients with neurologic dysfunction, whereas children with predominant glomerular damage are preferably diagnosed as having HUS. However, only about 40% of patients with acute TTP episodes have manifested the complete classic pentad[10]. All patients with HUS had an impairment of the kidneys, but renal abnormalities were also observed in 44% of patients with TTP; on the other hand, neurologic dysfunction was reported in 78% of patients with TTP, but also in one-third of patients with HUS[10]. Therefore, distinguishing between TTP and HUS clinically is difficult and somewhat arbitrary. TTP and HUS are often regarded as variants of one syndrome denoted as TTP/HUS. Since siblings of patients with TTP have been diagnosed to have HUS[11,12] and since acute episodes of TTP and HUS have been reported to occur sequentially even in the same patient, it has been suggested that the two disorders may be different manifestations of the same pathophysiologic process[13,14].

Epidemiology of thrombotic thrombocytopenic purpura and hemolytic uremic syndrome

TTP is believed to occur in 2–10 cases per million per year. In a study covering the 4523 TTP-associated deaths between 1968 and 1991 in the United States[15], the incidence of TTP was estimated to be approximately 3.7 cases per million residents, and the trend in mortality suggested that the incidence is increasing. Deaths were rare below the age of 20 years, and the mortality rate for patients older than 20 years increased steadily with increasing age. Numerous epidemic outbreaks of HUS in children have been reported[16], especially during the warmer months. The average annual incidence in the United States is about 1–2 per million, but it may be several times greater in some other countries, e.g. Scotland[17] or Argentina[18].

Pathogenesis of thrombotic thrombocytopenic purpura

The hypotheses concerning the etiology of TTP are controversial and suggest different pathogenetic mechanisms being responsible for development of the disorder[7,8]. Microvascular endothelial injury is generally considered as the primary event. Most commonly, patients with TTP have no discernible underlying disease or pathologic condition. However, in some patients TTP has been associated with the presence in plasma of platelet aggregating agents, viral or bacterial infection, bone marrow transplantation, cancer and cancer chemotherapy, use of the antiplatelet agent ticlopidine, alcohol abuse, pregnancy, and autoimmune disorders. Oxidative injury, free radical formation, reduced production of prostacyclin (PGI_2), impaired fibrinolysis and abnormal processing of von Willebrand factor (vWF) multimers have been proposed to be pathogenetically relevant for the development of TTP.

Platelet aggregating agents in thrombotic thrombocytopenic purpura

Several groups have attempted to identify in plasma samples from patients with TTP, a platelet aggregating factor responsible for formation of disseminated microvascular platelet thrombi. A 37 kD protein that caused aggregation of normal platelets was isolated from plasma of patients with acute TTP[19].

A calcium-dependent cysteine protease, capable of inducing platelet aggregation, was established in the sera of 15 patients with TTP during the acute phase of the disease but not in remission[20]. Moore et al.[21] have shown that the calpain from human platelets degraded vWF subunits to 205 kD and 85 kD fragments, with complete loss of large vWF multimers; calpain-degraded vWF was found to bind to platelet glycoproteins IIb/IIIa on activated platelets and to cause their aggregation. Active calpain in plasma of patients with TTP was associated with platelet microparticles protecting it from plasma inhibitors[22] such as α_2-macroglobulin and high molecular weight kininogen. While purified calpain is readily inhibited by plasma from normal subjects, there was no inhibition of microparticle-bound calpain from TTP patients by normal plasma[22]. It appears that microparticles released from thrombin-activated normal platelets, displaying procoagulant phospholipids for the process of blood coagulation, are different from those found in patients with TTP, since there was no calpain activity present in the serum of control subjects, in contrast to serum from TTP patients. This lack of calpain containing microparticles in the serum of normal individuals cannot be explained by their consumption in the fibrin clot, because the same levels of calpain activity were found in plasma and in serum of patients with TTP[22].

Unusually large von Willebrand factor in thrombotic thrombocytopenic purpura

Unusually large vWF multimers have been observed in plasma from patients with chronic relapsing forms of TTP[23,24] and HUS[25]. vWF is a multimeric plasma glycoprotein synthesized in endothelial cells and megakaryocytes. vWF mediates the initial platelet adhesion to the subendothelium of the damaged vessel wall at high shear rates. Only the highly polymeric forms of vWF are hemostatically active. While the circulating vWF in normal plasma shows only weak binding affinity for platelets, these interactions can be amplified by non-physiologic modulators, such as ristocetin and botrocetin. Electron microscopic studies[26] showed that about 90% of vWF in a resting solution is present in the form of linear polymers coiled upon itself in a ‘ball of yarn’ form. It is conceivable that the vWF undergoes a shear stress-induced conformational transition from a globular state to an extended chain conformation with exposure of intramolecular globular domains[27]. At sites of high fluid shear stress, encountered in arterioles and capillaries, the large vWF filaments apparently unfold and establish multiple interactions with platelets. Even though the affinity of the individual vWF subunits for platelet receptors is very low, multiple interactions of repeating binding sites on extended filaments of vWF with receptor glycoproteins Ib on the platelet surface may dramatically increase the stability of the bonds between platelet and vWF, and thus lead to platelet agglutination. Binding affinities of the large vWF multimers for platelets were shown to be up to ten times higher than those of the smaller molecular forms of vWF[28].

From the storage organelles (Weibel–Palade bodies) of the activated endothelial cells, vWF is secreted in the form of extremely large vWF multimers[29,30]. The length of these unusually large polymeric filaments, if stretched, would surpass the diameter of an intact platelet. These very large vWF multimers, released from the endothelial cells, are even more effective than the largest vWF forms, circulating in normal plasma, in inducing platelet agglutination under conditions of high fluid shear[31].

Cambridge Books Online © Cambridge University Press, 2009

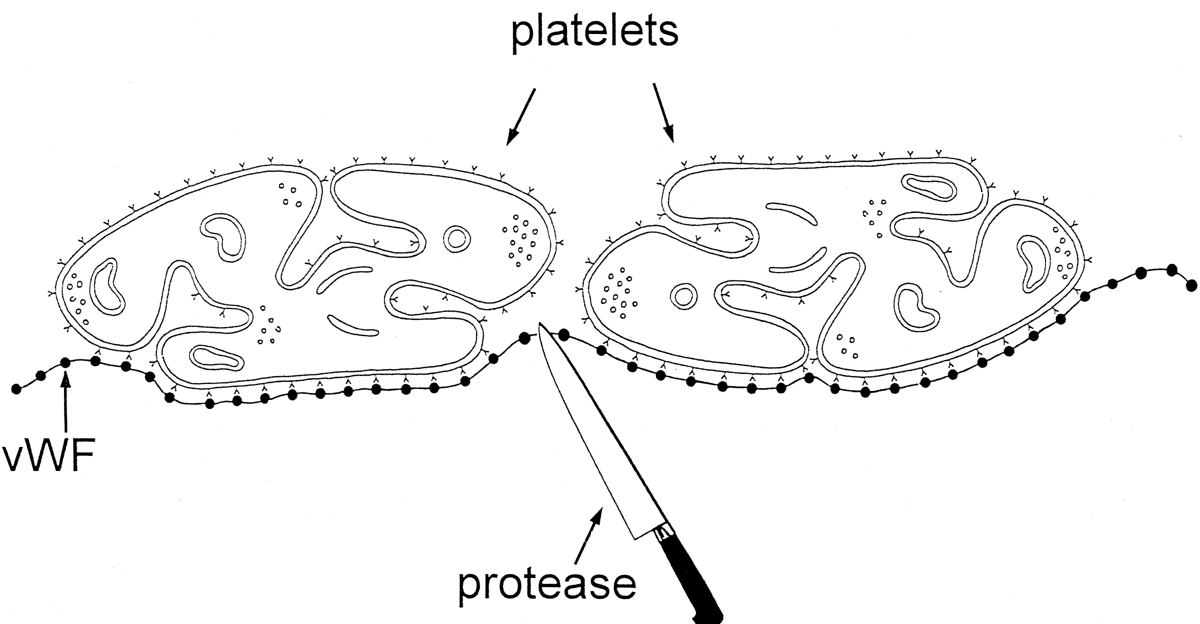


Fig. 40.1. Aggregation of platelets by unusually large multimers of vWF. In this model, the extended filament of an extremely large vWF multimer adheres to and joins a pair of circulating platelets. Multiple interactions of vWF with platelet glycoprotein Ib lead to an increased binding affinity that is required to resist high shear forces in the blood circulation. Cleavage of vWF multimers by a protease leads to smaller forms of vWF having an impaired ability to adhere firmly to the surface of two platelets.

Deficiency of vWF-cleaving protease in thrombotic thrombocytopenic purpura

Multimeric vWF is slowly but continuously attacked by protease(s) in the circulation[30,32–34]. Proteolytic cleavage of the vWF subunit between residues tyrosine at position 842 and methionine at position 843 is responsible for the post-translational processing of vWF[32].

Moake et al.[23] suggested that the presence of unusually large vWF multimers in plasma of patients with chronic relapsing forms of TTP may be due to an excessive release of vWF from endothelial cells and/or to an impaired degradation of the highly multimeric forms of vWF by a 'depolymerase'. A specific protease (Fig. 40.1), cleaving purified vWF in vitro to the same fragments as those produced in vivo, has been isolated from normal human plasma[35,36]. This protease is not inhibited by inhibitors of serine proteases, cysteine proteases, or matrix metalloproteases. It requires calcium ions (or barium ions) for full activity, but it is different from calpain. Normal human vWF appears to be resistant against proteolytic cleavage in a physiologic saline solution but becomes susceptible to degradation at low ionic strength or in the presence of 1.0–1.5 mol/l urea[35] or 1.1–1.2 mol/l guanidinium chloride[36]. Tsai et al.[37] also observed that the proteolytic degradation was enhanced under conditions of high fluid shear stress. It is apparent that the hypotonic salt concentration, urea, guanidinium chloride or shear stress cause a conformational change in the vWF molecule resulting in exposure of the cleavage site 842 Tyr–843 Met.

Impaired degradation of vWF, that had been suspected in patients with TTP[23], was first established in two brothers with chronic relapsing TT by Furlan et al.[38], who confirmed the presence in plasma of unusually large vWF multimers. The latter were absent in plasma samples of their asymptomatic parents and sister. Both affected patients showed complete deficiency of vWF-cleaving protease activity, whereas their parents had about half normal protease activity, and the sister had normal activity[38]. No inhibition of the protease activity in normal plasma was found after incubation with an equal volume of patient plasma. The in vivo recovery of vWF-cleaving protease following plasma exchange was about 100% and its biologic half-life was 2–4 days[39]. This half-life is uniquely long for a proteolytic enzyme, since the majority of proteases are rapidly inactivated in plasma by circulating inhibitors; thus, the activated forms of most proteases have plasma half-lives measured in seconds to minutes. It appears that, in patients with recurring TTP and severe congenital deficiency of vWF-cleaving protease, plasma exchange or even

Cambridge Books Online © Cambridge University Press, 2009

plasma infusion alone may be effective in suppressing the acute TTP episodes and in preventing TTP relapses[39].

The lack of vWF-cleaving protease activity in another patient with recurrent severe episodes of TTP was shown to be due to an acquired inhibitor of the protease activity[40]. Treatment with plasma, corticosteroids and vincristine led to a transient disappearance of the autoantibody and appearance of the protease activity. Three months after remission from the initial TTP event, the protease inhibitor returned, vWF-cleaving protease activity disappeared, and the platelet count gradually decreased. Severe relapses of TTP occurred 7 and 11 months after the first acute TTP event. Treatment with vincristine, that had been recommended for plasma-resistant patients[41–43], resulted in a transitory improvement of the platelet count even though the inhibitor did not vanish[40], suggesting a direct effect of vincristine on platelets rather than an immunosuppressive mode of action. An increased titre of the autoantibody was noted following treatment with fresh frozen plasma (FFP)[40]. This observation suggests that plasma exchange and replacement with FFP alone may not be sufficient in patients with acquired TTP. Splenectomy, performed 1 year after the first TTP event, resulted in disappearance of the autoantibody and normalization of the protease activity and of the platelet count[40]. No clinical relapse occurred and no protease inhibitor was detected as of the last visit, more than 4 years following splenectomy. The therapeutic effect of splenectomy appears to be due to removal of the B cells responsible for production of autoantibodies inhibiting vWF-cleaving protease, and corroborates the empirically observed beneficial effect of splenectomy in patients with TTP[44–46]. It is conceivable that splenectomy should primarily be reserved for plasma-refractory patients showing an antibody against vWF-cleaving protease.

Association of TTP with constitutional as well as with acquired deficiency of vWF-cleaving protease was subsequently confirmed in a multicentre retrospective study on the prevalence of protease deficiency in patients with familial and acquired TTP/HUS[47]. Lacking or strongly decreased protease activity ($<$5% of normal plasma) was found in 26 of 30 examined patients during an acute TTP event (6 familial and 24 non-familial TTP cases) (Fig. 40.2) while the protease activity was higher than 50% of pooled normal plasma in 120 healthy subjects. An inhibitor of vWF-cleaving protease was established in 20 of 24 patients with non-familial TTP but in none with familial TTP. The protease inhibitor is an IgG autoantibody that usually disappears, at least temporarily, in remission. Normal protease activity was found in 21 of 23 patients with an acute episode of HUS (10 familial and 13 non- familial cases). The difference in vWF-cleaving protease activity between TTP and HUS permits a differential diagnosis of these two similar disorders that are often difficult to distinguish clinically.

The acquired deficiency of vWF-cleaving protease in patients with TTP was independently confirmed by Tsai and Lian[48], who found no activity in plasma samples of 37 patients during the acute episode. IgG antibodies with protease inhibitory activity were detected in two-thirds of samples collected during the acute event. No inhibitor was found in 16 plasma samples obtained during the remission of TTP, or in 74 plasma samples from normal subjects or patients with hemolysis, thrombocytopenia or thrombosis from other causes[48]. Complete absence or severe reduction of vWF-cleaving protease activity was described in seven patients with ticlopidine-associated TTP[49]. An inhibitor of the protease was found in 6 of these patients within 2 to 7 weeks after the initiation of ticlopidine therapy. The protease deficiency resolved after ticlopidine therapy had been discontinued and plasmapheresis instituted. vWF-cleaving protease was assayed in 2 of 11 patients with clopidogrel-associated TTP[50]; in both patients, vWF-cleaving protease was undetectable during the acute episodes of TTP, and IgG inhibitors of the protease were present.

In contrast to the above patients with autoimmune deficiency of vWF-cleaving protease, normal protease activities were found in patients with bone marrow transplantation (BMT)-associated TTP[51]. It appears that BMT-associated thrombotic microangiopathy should be classified as atypical HUS, in which the protease activity is usually in the normal range, rather than as TTP. It has been recently reported by Mammucci et al.[52] that the activity of vWF-cleaving protease may be depressed below the normal range (50–200%) in healthy newborns and in pregnant women during the third trimester, in patients with liver cirrhosis or inflammatory diseases. Therefore, only complete deficiency of vWF-cleaving protease should be regarded as a reliable marker for TTP.

Assays of vWF-cleaving protease

Immunoblotting of degraded vWF substrate following electrophoresis in SDS-agarose gel

Purified vWF, prepared from cryoprecipitate of normal human plasma by gel filtration on Sepharose CL-2B, is used as substrate for protease activity[35]. The protease in diluted test plasma is activated with barium ions. The reaction mixture is dialysed using a hydrophilic filter floating on the surface of the dialysis solution (1.5 mol/l urea,

Cambridge Books Online © Cambridge University Press, 2009

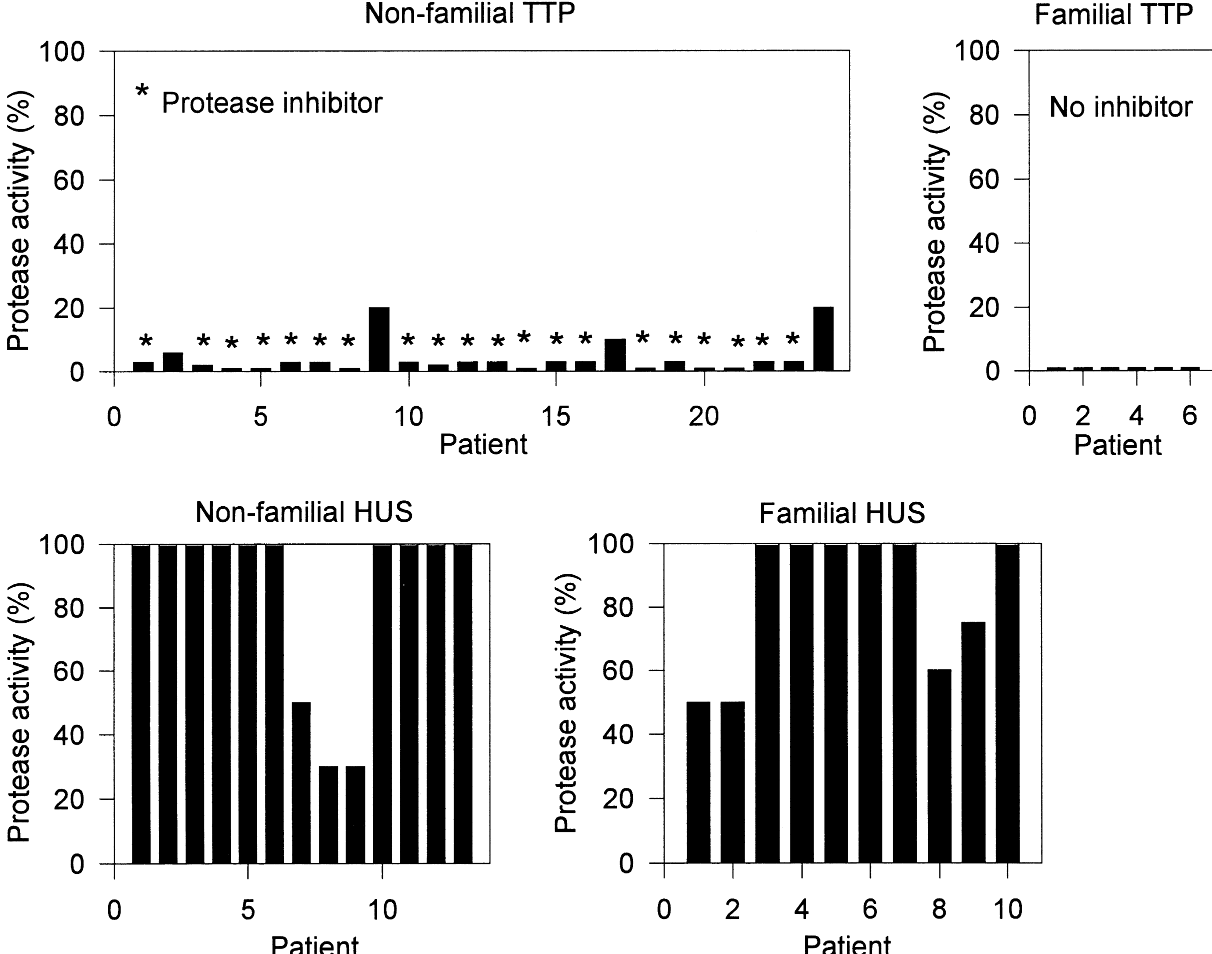


Fig. 40.2. Activity of vWF-cleaving protease in patients with TTP or HUS. In 20 of 24 patients with non-familial TTP, an inhibitor of vWF-cleaving protease was found, whereas no patient with familial TTP had an inhibitor. None of 13 patients with non-familial HUS or 10 patients with familial HUS had a severe deficiency (<5%) of vWF-cleaving protease which was found in 26 of 30 patients with TTP.

5 mmol/l Tris, pH 8.0) for 24 h at 37 °C. The reaction is stopped by addition of EDTA, and the extent of vWF degradation is assessed by multimer analysis in SDS–1.4% agarose gel. After electrophoresis, the proteins are electroblotted to nitrocellulose and stained with peroxidase-conjugated rabbit antibodies against human vWF. The method is very sensitive in the range of low protease activity and can discriminate between 0% and 1% activity. On the other hand, its accuracy is only moderate in the subnormal and normal range of protease activity. The inhibitor assay is performed by measuring the residual activity in the normal plasma after preincubation with the patient plasma[40].

Analysis of protease-digested vWF substrate by SDS-polyacrylamide gel electrophoresis (PAGE)

Purified vWF is preincubated in 1.5 mol/l guanidine and used as substrate for the protease assay[48]. After incubation of vWF substrate with diluted patient plasma in the presence of calcium chloride for 1 h at 37 °C, the reaction is terminated by addition of SDS and EDTA. The resulting fragments of vWF are electrophoresed in SDS–6% PAGE under non-reducing conditions. Following electrotransfer to nitrocellulose, vWF is visualized by probing the blots with ^{125}I-labelled rabbit IgG against human vWF and autoradiography. This assay is based on the generation, from the purified vWF, of dimers of carboxy terminal 176 kD fragments and of dimers of amino terminal 140 kD frag-

Cambridge Books Online © Cambridge University Press, 2009

ments, migrating in the SDS–PAGE as 350 kD and 200 kD bands, respectively. The radioactivity of the 350 kD band, measured by optical densitometry of the autoradiograms, represents the protease activity in the test sample.

Collagen binding assay for quantitating the proteolysis of vWF

Both assays of vWF-cleaving protease activity described above are very cumbersome and require expertise available only in laboratories familiar with immunoblotting. In a recently described method[53], the electrophoretic analysis of vWF multimers or its fragments is replaced by an ELISA based on preferential binding of large vWF multimers to collagen. This method also avoids preparation of protease-free vWF: purified vWF is replaced as substrate by normal plasma in which the vWF-cleaving protease has been irreversibly inactivated by 3 h exposure to EDTA. EDTA in the substrate plasma preparation is then removed by exhaustive dialysis against 5 mmol/l Tris, pH 8.0. 1.5 mol/l urea. Incubation with diluted test plasma samples is performed in the presence of urea and barium ions for 2 h at 37 °C, and the reaction is terminated by addition of sodium sulfate. The binding of degraded vWF to microtitre plates coated with collagen type III is measured using a peroxidase-conjugated anti-vWF antibody. The assay can be completed within 6 hours, requires no complex equipment and can be performed in a routine hospital laboratory.

Assay of vWF-cleaving protease using monoclonal antibodies to vWF

Another approach to measuring vWF-cleaving protease has been described by Obert et al.[54]. Wild-type recombinant vWF, devoid of vWF-cleaving protease, is used as substrate. The protease in test plasma samples is activated with barium ions and incubated with vWF substrate for 18 h at 22 °C on filter membranes floating on the top of 5 mmol/l Tris buffer containing 1.5 mol/l urea. The extent of vWF degradation is estimated by a two-site immunoradiometric assay, using the monoclonal antibody to a C-terminal epitope of vWF as the coating antibody, and a mixture of ^{125}I-labelled monoclonal antibodies to N-terminal epitopes as second antibody.

The latter three methods are more accurate in the normal and subnormal range but less sensitive in the very low range of protease activity than SDS-agarose electrophoresis of degraded vWF multimers. A simplified scheme for differential diagnosis of TTP, following the assay of vWF-cleaving protease, is presented in Fig. 40.3. Patients with signs and symptoms of TTP/HUS, who have no measurable protease activity during the acute episode, may be diagnosed as having classic TTP, whereas normal activity is compatible with the diagnosis of HUS. Since the half-time of vWF-cleaving protease in patients without inhibitor is 2–4 days[39], the blood sample has to be collected before, or at least 10 days after any plasma treatment. Patients with congenital protease deficiency are protease deficient during the acute TTP event as well as in the remission. Their platelet count is normalized within 2–3 days after a single plasma infusion, without any additional treatment, but tends to decrease 1–2 weeks later. On the other hand, patients with acquired TTP, due to an autoantibody, will hardly recover after a single plasma treatment. Immune suppression and other supportive measures lead to disappearance of the inhibitor and to reappearance of vWF-cleaving protease in the remission. Therefore, normal protease activity from a patient in remission does not exclude an acquired form of TTP during the preceding acute episode.

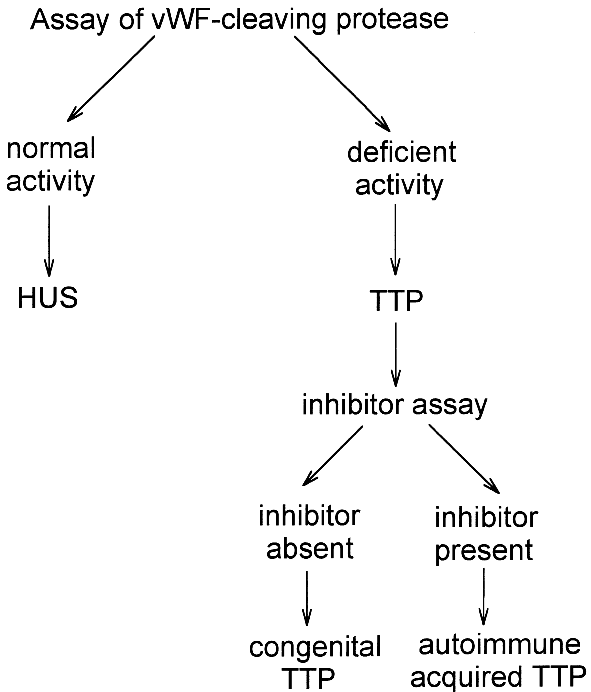


Fig. 40.3. Differential diagnosis in patients with TTP/HUS. Interpretation of results obtained by the assays of vWF-cleaving protease and of its inhibitor has to be performed with appropriate consideration of the clinical data.

Cambridge Books Online © Cambridge University Press, 2009

Pathophysiologic mechanisms of hemolytic uremic syndrome

The question regarding the involvement of unusually large vWF multimers in the pathogenesis of HUS remains open. Infection by shiga toxin-producing *Escherichia coli* (most common serotype O157: H7) has been recognized as the predominant etiology of HUS in children. In these cases, endothelial damage induced by shiga-like verotoxins is widely assumed to be the primary cause of renal dysfunction. Verocytotoxin was shown to be cytopathic to renal microvascular endothelial cells in culture, supporting the hypothesis that the vasculopathy of HUS is caused directly by the toxic action of verotoxins on endothelial cells[55,56]. Uchida et al.[57] showed the deposition of shiga toxins in the distal tubular epithelium of the kidney. Distal tubular epithelial cells were found to express a functional receptor (Gb3/CD77) for shiga toxins and to be susceptible to its cytotoxicity[58]. Verotoxin 1 was internalized in cultured human glomerular epithelial cells, mesangial cells and cortical tubular epithelial cells[59]. These observations suggest that endothelial cells may not be the sole target for shiga toxin-mediated cell injury.

According to the tentative scheme of the pathogenesis of enterohemorrhagic *E. coli*-induced HUS, proposed by Taylor & Monnens[60], the initial reaction is binding of the toxin to the monocytes followed by release of interleukins IL-1β, IL-6, IL-8, and tumour necrosis factor-α (TNFα). Thus, inflammatory mediators, locally produced by toxin-stimulated monocytes, may contribute to the pathogenesis of HUS[61]. Moreover, incubation of verotoxin-loaded polymorphonuclear leukocytes (PMNs) with human glomerular microvascular endothelial cells resulted in toxin transfer to the endothelial cells, inhibition of protein synthesis and cell death[62], suggesting that PMNs participate in the development of acute HUS.

Reduced serum levels of the third component (C3) of the complement system have been reported in familial and atypical HUS[63,64]. It appears that decreased serum concentrations of C3 in patients with hereditary or sporadic HUS are due to the deficiency or a functional abnormality of the complement factor H[65–68], the most important plasma regulator of the alternative complement activation pathway. Thus, a complement-mediated mechanism of endothelial cell activation, leading to massive release of unusually large vWF multimers, seems to be a plausible hypothesis of familial and sporadic HUS etiology.

vWF-cleaving protease deficiency alone is not sufficient to cause clinical thrombotic thrombocytopenic purpura

The age distribution of patients with constitutional protease deficiency, shown in Fig. 40.4 (M. Furlan, unpublished data), demonstrates a striking age-dependent clustering of the first acute TTP episode. One-half of the 20 patients with complete constitutional deficiency of vWF-cleaving protease had their first TTP event between the neonatal period and the age of 5 years; this acute episode was then often followed by chronic relapsing TTP unless the patients received prophylactic plasma therapy at regular intervals of about 3 weeks. The other half of the patients with severe constitutional protease deficiency remained apparently asymptomatic until the age of about 20–30 years. Two sisters with hereditary protease deficiency (denoted in Fig. 40.4 as patients 14 and 15) had their first acute TTP episode during their first pregnancies while their brother, aged 44 years, has never had any symptoms of TTP. Similarly, another pair of sisters (patients 13 and 18) were asymptomatic until their first pregnancy; they also have a protease-deficient brother, aged 37 years, who has never had an acute event of TTP.

These results indicate that most, if not all, patients with classic TTP have severe protease deficiency but not all individuals with constitutional protease deficiency develop acute TTP. The first acute episode of TTP in a patient with constitutional deficiency of vWF-cleaving protease tends to be followed by a chronic relapsing form of the disease. Patient 11 had no signs or symptoms of TTP before the age of 20 years, but experienced several relapses in the years following the first acute bout of TTP (M. Furlan, unpublished data). The last acute episode, at the age of 36 years, led to the institution of regular plasma infusion, starting with 2 units of FFP every third week, as recommended elsewhere[3,69–72]. The estimated initial concentration of vWF-cleaving protease in patient 11, after infusion of 2 units of FFP, was estimated to be about 15% of normal plasma. The platelet count in this patient increased steadily during the first 10 days after each plasma infusion, although the circulating protease concentration had been reduced to less than 5% within the same time interval (M. Furlan, unpublished data). We conclude tentatively from this observation that 5% protease activity may be sufficient to degrade the very large and adhesive vWF multimers to smaller vWF forms, which do not spontaneously agglutinate platelets. Since the kinetics of platelet consumption at very low protease concentrations appears to vary from patient to patient, we suggest that, in each patient with constitutional protease deficiency, the optimum infusion intervals

Cambridge Books Online © Cambridge University Press, 2009

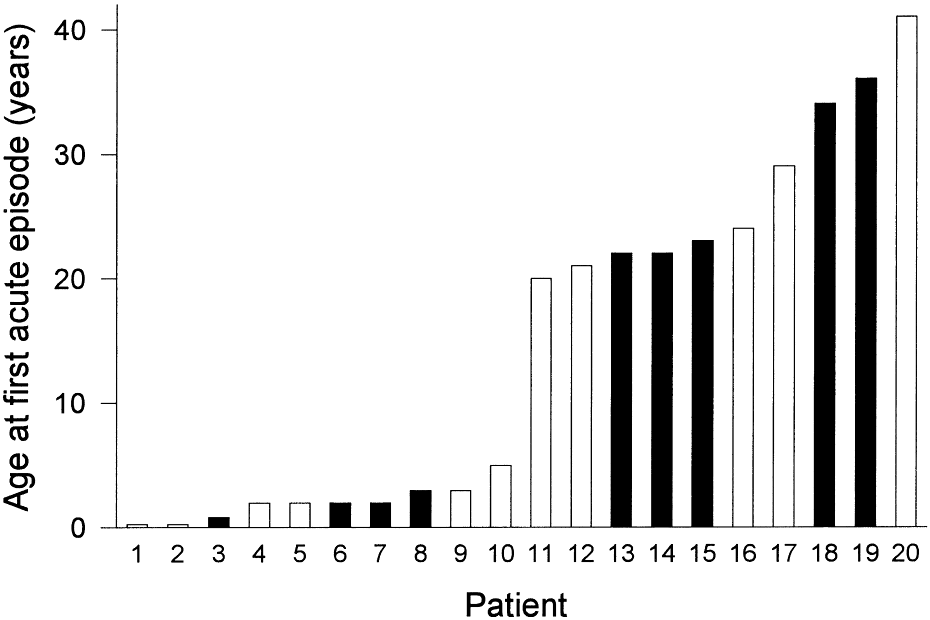


Fig. 40.4. Age distribution of the first acute TTP episode in 20 patients with complete congenital deficiency of vWF-cleaving protease. Empty bars, male patients; full bars, female patients.

and the infused plasma volumes be empirically established from the individual time course of the platelet count.

Data presented in Fig. 40.4 suggest that the prevalence of congenital vWF-cleaving protease deficiency may be higher than diagnosed, since some severe protease-deficiencies remain undetected in asymptomatic subjects. It is not improbable that individuals with an acquired protease deficiency may also escape detection, because the autoantibodies against the protease may disappear before the development of an acute TTP. As reported elsewhere[40], a patient with acquired TTP had his first relapse 3 months after the antibody-mediated disappearance of the vWF-cleaving protease, suggesting that a specific pathophysiologic condition was required to trigger the onset of the acute episode. It was also observed[47] that some patients with acquired TTP progressed to remission before the complete disappearance of the inhibitor and normalization of the vWF-cleaving protease. The above considerations regarding the occurrence of acute TTP in severely protease-deficient patients are reminiscent of venous thrombophilia in patients with genetic (e.g. factor V Leiden, deficiencies of antithrombin III, proteins C and S) or acquired (antiphospholipid antibodies, oral contraceptives, pregnancy, trauma, surgery, malignancy, previous thrombotic episodes) risk factors: a single risk factor alone is hardly sufficient to induce venous thrombosis in these patients, but the likelihood of an acute thrombotic event increases if several risk factors accumulate and recurrence rate is high after a first thromboembolic event.

It remains unclear why some individuals with severe congenital protease deficiency experience acute episode(s) of TTP during childhood while others remain asymptomatic for decades, as shown in Fig. 40.4. The vWF-cleaving protease deficiency is a very strong risk factor for TTP, but the development of an acute thrombotic microangiopathy requires a trigger, such as pregnancy, alcohol abuse, viral or bacterial infection. It is generally assumed that this trigger leads to activation or death of the microvascular endothelial cells releasing the unusually large vWF multimers from the storage organelles.

Antibodies against platelets and endothelial cells in patients with thrombotic thrombocytopenic purpura

Several attempts have been undertaken to pinpoint the mechanism of endothelial cell damage and death. Lytic antiendothelial cell antibodies were detected in plasma of patients with TTP[73] and HUS[74]. Endothelial cell-reactive plasmas were found to induce aggregation of human platelets in vitro[73], suggesting that TTP plasma may account for both endothelial cell damage and direct thrombotic vascu-

Cambridge Books Online © Cambridge University Press, 2009

lar occlusion in TTP. More recently, much attention has been devoted to the presence in TTP of antibodies directed against the human CD36[75,76], an integral membrane protein (also known as platelet glycoprotein IV), expressed by platelets as well as by some other cells including endothelial cells. It is interesting that CD36 expression on endothelial cells is restricted to capillary endothelial cells and is not observed on large vessel endothelial cells[77]. CD36 is the receptor of the platelet-agglutinating 37 kD protein[78], a previously recognized platelet aggregating factor present in plasmas of patients with TTP[19].

It is unknown whether antiendothelial cell and antiplatelet autoantibodies are directly involved in formation of microvascular platelet aggregates or whether they rather induce damage or apoptosis of endothelial cells and subsequent release of very large vWF multimers from intracellular stores. Antibodies against endothelial cells and platelets might arise as a secondary response of the immune system to inflammatory events in TTP patients, leading to expression of cellular neoantigens or exposure of cryptoantigens[79]. Antibodies against endothelial cells were also found in patients with disorders unrelated to TTP[80]. A recent study suggested that antibodies reactive against platelet or endothelial cell antigens are not prevalent in TTP, and that more than a third of reactive antibodies were human leukocyte antigen alloantibodies[81]. In addition, complement is not depleted in acute TTP[82], a feature that would be expected if circulating antibodies, directly toxic to endothelial cells, were involved. Thus, the evidence that antibody-mediated injury of endothelial cells is a primary etiologic factor in TTP remains controversial.

Inflammatory cytokines and apoptosis of endothelial cells in thrombotic thrombocytopenic purpura and the hemolytic uremic syndrome

Ultrastructural alterations in microvascular endothelial cells have been found in TTP by electron microscopy showing cytoplasmic vacuoles and swollen mitochondria. These changes, reflecting early apoptotic lesions, have been noted even in the absence of platelet microthrombi, suggesting that they are not simply a consequence of vessel occlusion. Plasmas of patients with either idiopathic TTP or HIV-associated microangiopathy were shown to induce apoptosis of primary human endothelial cells of dermal microvascular but not of umbilical vein origin[83]. Detachment of endothelial cells from renal microvasculature and their appearance in the circulation[84] are consistent with an apoptotic process. Furthermore, plasmas from TTP and sporadic HUS patients, but not from D+ diarrhea positive HUS patients, induced apoptosis and expression of the apoptosis-associated protein Fas (CD95), a type I plasma membrane receptor involved in the regulation of programmed cell death, in microvascular endothelial cells of renal, cerebral and dermal origin but not in those of pulmonary and hepatic origin[85]. This difference parallels the in vivo pathology of TTP/sporadic HUS, with notable sparing of the pulmonary and hepatic microvasculature.

The primary apoptosis-inducing agents have not yet been identified. It has been tentatively proposed that the impaired endothelial cell survival might be associated with the loss of extracellular matrix proteins[86]. Cytokines might also play a pathogenetic role. Fas was shown to become upregulated on endothelial cells by exposure to TNF-α[83] and its upregulation was accompanied by induction of the cell cycle protein Cdc2 in the dermal microvascular endothelial cells, but not in large vessel endothelial cells[87]. However, induction of Fas by the reactive components of TTP and HUS plasmas could simply reflect an activated cell phenotype and be irrelevant to apoptosis[87]. TNF-α can initiate apoptosis of endothelial cells, but the TTP plasma-mediated apoptosis could not be blocked with anti-TNF-α antibodies[83]. The finding that TTP plasma induces endothelial cell apoptosis has recently been confirmed by other authors[88]; nevertheless, Fas did not seem to be involved. Karpman et al.[89] reported apoptosis of renal cortical cells in children with postenterohepatic (D+) HUS. They also found apoptosis in cultured renal tubular cells incubated with shiga-like toxin from *E. coli*. Interleukin-6 (IL-6) was elevated in the serum of 33 of 35 children with HUS (31 diarrhea-associated and 4 non-diarrhea-associated) and in 2 of 2 children with recurrent TTP[90]; the latter two patients have been found to have constitutional protease deficiency. The same study showed increased levels of TNF-α in the serum from 7 of 35 HUS patients and from both children with TTP. Circulating levels of pro-inflammatory IL-6 were also increased in another study of children with HUS due to *E. coli* infection; the concentrations of the anti-inflammatory IL-10 were also increased, but to a lesser extent than IL-6[91], suggesting that an imbalance of pro- and anti-inflammatory cytokines may be involved in the pathophysiology of verotoxin-associated HUS. IL-6 was found to activate normal human platelets in vitro[92]. In addition to the above, sometimes discordant models of the apoptotic endothelial cell death in TTP/HUS, another study reported that calpain is involved in apoptosis-like events in washed human platelets[93]. Although platelets were found to contain the pro-apoptotic caspases 3 and 9, it was found that calpain, and not

Cambridge Books Online © Cambridge University Press, 2009

caspases, promoted the apoptosis-like events in platelets during their activation.

Need for a new classification of thrombotic microangiopathies (TMA)

The progress in our understanding of the pathogenesis of TMAs in recent years calls for establishment of a new classification that would probably be helpful for therapeutic purposes. This classification should recognize the differences in the congenital and acquired (due to autoantibodies) deficiencies of vWF-cleaving protease. The classic TMA, thus far frequently denoted as idiopathic TTP, may be triggered by different precipitating factors such as pregnancy, infection or drugs (ticlopidine), and is usually mediated by an autoimmune process abolishing vWF-cleaving protease activity. Furthermore, the deficiency of the complement factor H is a special type of TMA including many cases of familial HUS. Diarrhea-associated HUS is a major well-established form that represent a distinctive class of TMA. In addition, bone marrow transplantation-associated, neoplasia-associated, and autoimmune disease-associated TMA appear to represent special types of TMA with similar clinical symptoms but different course and prognosis. A new classification should be helpful in devising the specific patient treatment which may be strikingly different in the different forms of TMA.

References

1 Moschcowitz E. Hyaline thrombosis of the terminal arterioles and capillaries: a hitherto undescribed disease. *Proc NY Path Soc* 1924; 24: 21–24.
2 Moschcowitz E. An acute febrile pleiochromic anemia with hyaline thrombosis of the terminal arterioles and capillaries. An undescribed disease. *Arch Int Med* 1925; 36: 89–93.
3 Schulman I, Pierce M, Lukens A et al. Studies on thrombopoiesis. I. A factor in normal human plasma required for platelet production; chronic thrombocytopenia due to its deficiency. *Blood* 1960; 16: 943–957.
4 Upshaw JD. Congenital deficiency of a factor in normal plasma that reverses microangiopathic hemolysis and thrombocytopenia. *N Engl J Med* 1978; 298: 1350–1352.
5 Wallace DC, Lovric A, Clubb JS et al. Thrombotic thrombocytopenic purpura in four siblings. *Am J Med* 1975; 38: 724–734.
6 Fuchs WE, George JN, Dotin LN et al. Thrombotic thrombocytopenic purpura. Occurrence two years apart during late pregnancy in two sisters. *J Am Med Assn* 1976; 235: 2126–2127.
7 Ruggenenti P, Remuzzi G. The pathophysiology and management of thrombotic thrombocytopenic purpura. *Eur J Haemat* 1996; 56: 191–207.
8 Moake JL. Studies on the pathophysiology of thrombotic thrombocytopenic purpura. *Semin Hemat* 1997; 34: 83–89.
9 Gasser C, Gauter E, Steck A, Siebenmann RE, Oechslin R. Hämolytisch-urämische Syndrome: bilaterale Nierenrindennekrosen bei akuten erworbenen hämolytischen Anämien. *Schweiz Med Wochenschr* 1955; 85: 905–909.
10 Thompson CE, Damon LE, Ries CA et al. Thrombotic microangiopathies in the 80s: clinical features, response to treatment, and the impact of the human immunodeficiency virus epidemic. *Blood* 1992; 80: 1890–1895.
11 Hellman RM, Jackson DV, Buss DH. Thrombotic thrombocytopenic purpura and hemolytic-uremic syndrome in HLA-identical siblings. *Ann Int Med* 1980; 93: 283–284.
12 Elias M, Horowitz J, Tal I et al. Thrombotic thrombocytopenic purpura and haemolytic uremic syndrome in three siblings. *Arch Dis Childh* 1988; 63: 644–646.
13 Remuzzi G, Garella S. HUS and TTP: variable expression of a single entity. *Kidney Int* 1987; 32: 292–308.
14 Ruggenenti P, Remuzzi G, Rossi EC. Epidemiology of the hemolytic uremic syndrome. *N Engl J Med* 1991; 324: 1065–1066.
15 Török TJ, Holman RC, Chorba TL. Increasing mortality from thrombotic thrombocytopenic purpura in the United States – analysis of national mortality data, 1968–1991. *Am J Hemat* 1995; 50: 84–90.
16 Gordjani N, Sutor AH, Zimmerhackl LB, Brandis M. Hemolytic uremic syndromes in childhood. *Semin Thromb Haemost* 1997; 23: 281–293.
17 Dundas S, Murphy J, Soutar RL et al. Effectiveness of therapeutic plasma exchange in the 1996 Lanarkshire *Escherichia coli* 0157:H7 outbreak. *Lancet* 1999; 354: 1327–1330.
18 López EL, Diaz M, Grinstein S et al. Hemolytic uremic syndrome and diarrhea in Argentine children: the role of Shiga-like toxin. *J Infect Dis* 1989; 160: 469–475.
19 Siddiqui FA, Lian EC-Y. Novel platelet-agglutinating protein from a thrombotic thrombocytopenic purpura plasma. *J Clin Invest* 1985; 76: 1330–1337.
20 Murphy WG, Moore JC, Kelton JG. Calcium-dependent cysteine protease activity in the sera of patients with thrombotic thrombocytopenic purpura. *Blood* 1987; 70: 1683–1687.
21 Moore JC, Murphy WG, Kelton JG. Calpain proteolysis of von Willebrand factor enhances its binding to platelet membrane glycoprotein IIb/IIIa: an explanation for platelet aggregation in thrombotic thrombocytopenic purpura. *Br J Haemat* 1990; 74: 457–464.
22 Kelton JG, Warkentin TE, Hayward CPM et al. Calpain activity in patients with thrombotic thrombocytopenic purpura is associated with platelet microparticles. *Blood* 1992; 80: 2246–2251.
23 Moake JL, Rudy CK, Troll JH et al. Unusually large plasma factor VIII: von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. *N Engl J Med* 1982: 307: 1432–1435.
24 Mannucci PM, Lombardi R, Lattuada A et al. Enhanced prote-

Cambridge Books Online © Cambridge University Press, 2009

olysis of plasma von Willebrand factor in thrombotic thrombocytopenic purpura and the hemolytic uremic syndrome. *Blood* 1989; 74: 978–983.

25 Moake JL, Byrnes JJ, Troll JH et al. Abnormal VIII: von Willebrand factor patterns in the plasma of patients with the hemolytic–uremic syndrome. *Blood* 1984; 64: 592–598.

26 Slayter H, Loscalzo J, Bockenstedt P et al. Native conformation of human von Willebrand protein. Analysis by electon microscopy and quasi-elastic light scattering. *J Biol Chem* 1985; 260: 8559–8563.

27 Siedlecki CA, Lestini BJ, Kottke-Marchant K et al. Shear-dependent changes in the three-dimensional structure of human von Willebrand factor. *Blood* 1996; 88: 2939–2950.

28 Federici AB, Bader R, Pagani S et al. Binding of von Willebrand factor to glycoproteins Ib and IIb/IIIa complex: affinity is related to multimeric size. *Br J Haemat* 1989; 73: 93–99.

29 Sporn LA, Marder VJ, Wagner DD. Inducible secretion of large, biologically potent von Willebrand factor multimers. *Cell* 1986; 46: 185–190.

30 Tsai HM, Nagel RL, Hatcher VB et al. Multimeric composition of endothelial cell-derived von Willebrand factor. *Blood* 1989; 73: 2074–2076.

31 Moake JL, Turner NA, Stathopoulos NA et al. Involvement of large plasma von Willebrand factor (vWF) multimers and unusually large vWF forms derived from endothelial cells in shear stress-induced platelet aggregation. *J Clin Invest* 1986; 78: 1456–1461.

32 Dent JA, Berkowitz SD, Ware J et al. Identification of a cleavage site directing the immunochemical detection of molecular abnormalities in type IIA von Willebrand disease. *Proc Natl Acad Sci, USA* 1990; 87: 6306–6310.

33 Dent JA, Galbusera M, Ruggeri ZM. Heterogeneity of plasma von Willebrand factor multimers resulting from proteolysis of the constituent subunit. *J Clin Invest* 1991; 88: 774–782.

34 Furlan M, Robles R, Affolter D et al. Triplet structure of von Willebrand factor reflects proteolytic degradation of high molecular weight multimers. *Proc Natl Acad Sci, USA* 1993; 90: 7503–7507.

35 Furlan M, Robles R, Lämmle B. Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. *Blood* 1996; 87: 4223–4234.

36 Tsai HM. Physiological cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion. *Blood* 1996; 87: 4235–4244.

37 Tsai HM, Sussman II, Nagel RL. Shear stress enhances the proteolysis of von Willebrand factor in normal plasma. *Blood* 1994; 83: 2171–2179.

38 Furlan M, Robles R, Solenthaler M et al. Deficient activity of von Willebrand factor-cleaving protease in chronic relapsing thrombotic thrombocytopenic purpura. *Blood* 1997; 89: 3097–3103.

39 Furlan M, Robles R, Morselli B et al. Recovery and half-life of von Willebrand factor-cleaving protease after plasma therapy in patients with thrombotic thrombocytopenic purpura. *Thromb Haemost* 1999; 81: 8–13.

40 Furlan M, Robles R, Solenthaler M et al. Acquired deficiency of von Willebrand factor-cleaving protease in a patient with thrombotic thrombocytopenic purpura. *Blood* 1998; 91: 2839–2846.

41 Levin M, Grünwald HW. Use of vincristine in refractory thrombotic thrombocytopenic purpura. *Acta Haemat* 1991; 85: 37–40.

42 Bobbio-Pallavicini E, Porta C, Centurioni R et al. Vincristine sulfate for the treatment of thrombotic thrombocytopenic purpura refractory to plasma-exchange. *Eur J Haemat* 1994; 52: 222–226.

46 Ferrara F, Copia C, Annunziata M et al. Vincristine as salvage treatment for refractory thrombotic thrombocytopenic purpura. *Ann Hemat* 1999; 78: 521–523.

44 Höffkes HG, Weber F, Uppenkamp M et al. Recovery by splenectomy in patients with relapsed thrombotic thrombocytopenic purpura and treatment failure to plasma exchange. *Semin Thromb Haemost* 1995; 21: 161–165.

45 Crowther MA, Heddle N, Hayward CMP et al. Splenectomy done during hematologic remission to prevent relapse in patients with thrombotic thrombocytopenic purpura. *Ann Int Med* 1999; 125: 294–296.

46 Mant MJ, Turner AR, Ritchie DBC et al. Splenectomy during partial remission in thrombotic thrombocytopenic purpura with prolonged plasma exchange dependency. *Am J Hemat* 1999; 62: 56–57.

47 Furlan M, Robles R, Galbusera M et al. von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. *N Engl J Med* 1998; 339: 1578–1584.

48 Tsai HM, Lian ECY. Antibodies to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. *N Engl J Med* 1998; 339: 1585–1594.

49 Tsai HM, Rice L, Sarode R et al. Antibody inhibitors to von Willebrand factor metalloproteinase and increased binding of von Willebrand factor to platelets in ticlopidine-associated thrombotic thrombocytopenic purpura. *Ann Int Med* 2000; 132: 794–799.

50 Bennet CL, Connors JM, Carwile JM et al. Thrombotic thrombocytopenic purpura associated with clopidogrel. *N Engl J Med* 2000; 342: 1773–1777.

51 van der Plas RM, Schiphorst ME, Huizinga EG et al. von Willebrand factor proteolysis is deficient in classic, but not in bone marrow transplantation-associated, thrombotic thrombocytopenic purpura. *Blood* 1999; 93: 3798–3802.

52 Mannucci PM, Canciani MT, Forza I, Lussana F, Lattuada A, Rossi E. Changes in health and disease of the metalloprotease that cleaves von Willebrand factor. *Blood* 2001; 98: 2730–2735.

53 Gerritsen H, Turecek P, Schwarz HP, Lämmle B, Furlan M. Assay of von Willebrand factor (vWF)-cleaving protease based on decreased collagen binding affinity of degraded vWF. A tool for the diagnosis of thrombotic thrombocytopenic purpura (TTP). *Thromb Haemost* 1999; 82: 1386–1389.

54 Obert B, Tout H, Veyradier A, Fressinaud E, Meyer D, Girma JP. Estimation of the von Willebrand factor-cleaving protease in

Cambridge Books Online © Cambridge University Press, 2009

plasma using monoclonal antibodies to vWF. *Thromb Haemost* 1999; 82: 1382–1385.
55 Inward CD, Williams J, Chant I et al. Verocytotoxin-1 induces apoptosis in vero cells. *J Infect* 1995; 213–218.
56 Adler S, Bollu R. Glomerular endothelial cell injury mediated by shiga-like toxin. *Kidney Blood Press Res* 1998; 21: 13–21.
57 Uchida H, Kiyokawa N, Horie H et al. The detection of shiga toxins in the kidney of a patient with hemolytic uremic syndrome. *Pediat Res* 1999; 45: 133–137.
58 Kiyokawa H, Taguchi T, Mori T et al. Induction of apoptosis in normal human renal tubular epithelial cells by *Escherichia coli* shiga toxins 1 and 2. *J Infect Dis* 1998; 178: 178–184.
59 Williams JM, Boyd B, Nutikka A et al. A comparison of the effects of verocytotoxin-1 on primary human renal cell cultures. *Toxicol Lett* 1999; 105: 47–57.
60 Taylor CM, Monnens LAH. Advances in haemolytic uraemic syndrome. *Arch Dis Child* 1998; 78: 190–193.
61 van Setten PA, Monnens LAH, Verstraten RGG et al. Effects of verocytotoxin-1 on nonadherent human monocytes: binding characteristics, protein synthesis, and induction of cytokine release. *Blood* 1996; 88: 174–183.
62 te Loo DMWM, Monnens LAH, van der Velden TJAM et al. Binding and transfer of verocytotoxin by polymorphonuclear leukocytes in hemolytic uremic syndrome. *Blood* 2000; 95: 3396–3402.
63 Ohali M, Shalev H, Schlesinger M et al. Hypocomplementemic autosomal recessive hemolytic uremic syndrome with decreased factor H. *Pedia Nephrol* 1998; 12: 619–624.
64 Noris M, Ruggenenti P, Perna A et al. Hypocomplementemia discloses genetic predisposition to hemolytic uremic syndrome and thrombotic thrombocytopenic purpura: role of factor H abnormalities. *J Am Soc Nephrol* 1999; 10: 281–293.
65 Pichette V, Querin S, Schürch W et al. Familial hemolytic uremic syndrome and homozygous factor H deficiency. *Am J Kidney Dis* 1994; 24: 936–941.
66 Warwicker P, Goodship THJ, Donne RL et al. Genetic studies into inherited and sporadic hemolytic uremic syndrome. *Kidney Int* 1998; 53: 836–844.
67 Rougier N, Kazatchkine MD, Rougier JP et al. Human complement factor H deficiency associated with hemolytic uremic syndrome. *J Am Soc Nephrol* 1998; 9: 2318–2326.
68 Ying L, Katz Y, Schlesinger M et al. Complement factor H gene mutation associated with autosomal recessive atypical hemolytic uremic syndrome. *Am J Hum Genet* 1999; 65: 1538–1546.
69 Moake J, Chintagumpala M, Turner N et al. Solvent/detergent-treated plasma suppresses shear-induced platelet aggregation and prevents episodes of thrombotic thrombocytopenic purpura. *Blood* 1994: 84:490–497.
70 Maes P, Brichard B, Vermylen C et al. Chronic relapsing thrombotic thrombocytopenic purpura: three case reports and update of the pathogenesis and therapeutic modalities. *Eur J Pediat* 1998; 157: 468–472.
71 Horowtiz MS, Pehta JC. SD plasma in TTP and coagulation factor deficiencies for which no concentrates are available. *Vox Sanguinis* 1998; 74, Suppl. 1: 231–235.
72 Häberle J, Kehrel B, Ritter J et al. New strategies in diagnosis and treatment of thrombotic thrombocytopenic purpura: case report and review. *Eur J Pediat* 1999; 158: 883–887.
73 Burns ER, Zucker-Franklin D. Pathologic effects of plasma from patients with thrombotic thrombocytopenic purpura on platelets and cultured vascular endothelial cells. *Blood* 1982; 60: 1030–1037.
74 Leung DY, Moake JL, Havens PL et al. Lytic anti-endothelial cell antibodies in haemolytic-uraemic syndrome. *Lancet* 1998; 23: 183–186.
75 Tandon NN, Rock G, Jamieson GA. Anti-CD36 antibodies in thrombotic thrombocytopenic purpura. *Br J Haemat* 1994; 88: 816–825.
76 Schultz DR, Arnold PI, Jy W et al. Anti-CD36 autoantibodies in thrombotic thrombocytopenic purpura and other thrombotic disorders: identification of an 85kD form of CD36 as a target antigen. *Br J Haemat* 1998; 103: 849–857.
77 Swerlick RA, Lee KH, Wick TM et al. Human dermal microvascular endothelial but not human umbilical vein endothelial cells express CD36 in vivo and in vitro. *J Immunol* 1992; 148: 78–83.
78 Lian ECY, Siddiqui FA, Jamieson GA et al. Platelet agglutinating protein p37 causes platelet activation through its binding to membrane glycoprotein IV. *Thromb Haemost* 1991; 65: 102–106.
79 Rock GA. Management of thrombotic thrombocytopenic purpura. *Br J Haemat* 2000; 109: 496–507.
80 Rock G, Chauhan K, Jamieson GA et al. Anti-CD36 antibodies in patients with lupus anticoagulans and thrombotic complications. *Br J Haemat* 1994; 88: 878–880.
81 Raife TJ, Atkinson B, Aster RH et al. Minimal evidence of platelet and endothelial cell reactive antibodies in thrombotic thrombocytopenic purpura. *Am J Hemat* 1999; 62: 82–87.
82 Ucar A, Fernandez HF, Byrnes JJ et al. Thrombotic microangiopathy and retroviral infections: a 13 year experience. *Am J Hemat* 1994; 45: 304–309.
83 Laurence J, Mitra D, Steiner M et al. Plasma from patients with idiopathic and human immunodeficiency virus-associated thrombotic thrombocytopenic purpura induces apoptosis in microvascular endothelial cells. *Blood* 1996; 87: 3245–3254.
84 Lefevre P, George F, Durand JM et al. Detection of circulating endothelial cells in thrombotic thrombocytopenic purpura. *Thromb Haemost* 1993; 69: 522.
85 Mitra D, Jaffe EA, Weksler B et al. Thrombotic thrombocytopenic purpura and sporadic hemolytic-uremic syndrome plasmas induce apoptosis in restricted lineages of human microvascular endothelial cells. *Blood* 1997; 89: 1224–1234.
86 Dang CT, Magid MS, Weksler B et al. Enhanced endothelial cell apoptosis in splenic tissues of patients with thrombotic thrombocytopenic purpura. *Blood* 1999; 93: 1264–1270.
87 Laurence J, Mitra D. Apoptosis of microvascular endothelial cells in the pathophysiology of thrombotic thrombocytopenic purpura/sporadic hemolytic–uremic syndrome. *Semin Hemat* 1997; 34: 98–105.
88 Wu XW, Li QZ, Lian ECY. Plasma from a patient with

Cambridge Books Online © Cambridge University Press, 2009

thrombotic thrombocytopenic purpura induces endothelial cell apoptosis and platelet aggregation. *Thromb Res* 1999; 93: 79–87.

89 Karpman D, Håkansson A, Perez MTR et al. Apoptosis of renal cortical cells in the hemolytic-uremic syndrome: in vivo and in vitro studies. *Infec Immun* 1998; 66: 636–644.

90 Karpman D, Andreasson A, Thysell H et al. Cytokines in childhood hemolytic uremic syndrome and thrombotic thrombocytopenic purpura. *Pediat Nephrol* 1995; 9: 694–699.

91 Litalien C, Proulx F, Mariscalco MM et al. Circulating inflammatory cytokine levels in hemolytic uremic syndrome. *Pediat Nephrol* 1999; 13: 840–845.

92 Oleksowicz L, Mrowiec Z, Isaacs et al. Morphologic and ultrastructural evidence for interleukin-6 induced platelet activation. *Am J Hemat* 1995; 48: 92–99.

93 Wolf BB, Goldstein JC, Stennicke HR et al. Calpain functions in a caspase-independent manner to promote apoptosis-like events during platelet activation. *Blood* 1999; 94: 1683–1692.

Cambridge Books Online © Cambridge University Press, 2009

Thrombocytosis and thrombocythemia

Tiziano Barbui and Guido Finazzi

Division of Hematology, Ospedali Riuniti, Bergamo, Italy

Definition

A thrombocytosis is defined as a platelet count above the upper limit of the reference range[1]. By convention, the normal range of blood platelet count in humans is considered to be 150 to 400×10^9/l. It is likely that this wide variation includes healthy individuals who have modest degrees of reactive thrombocytosis, such as young women with iron deficiency secondary to excessive menstrual blood loss. Consequently, although this is not well documented, the upper limit of the normal range in truly healthy people may be less than 400×10^9/l. Platelet values in the upper normal range should be interpreted in the clinical context of the individual patient who should be followed for eventual clear diagnostic evaluation.

Table 41.1. Classification of thrombocytosis

Reactive thrombocytosis
Infectious or inflammatory diseases
Malignancy
Iron deficiency anemia, hemolytic anemia, acute blood loss
Splenectomy
Rebound effect after chemotherapy or immune thrombocytopenia
Clonal thrombocytosis
Essential thrombocythemia
Polycythemia vera
Idiopathic myelofibrosis
Chronic granulocytic leukemia
Myelodysplastic syndromes
5q- syndrome
Idiopathic refractory sideroblastic anemia

Classification and differential diagnosis

Based on the mechanisms inducing the increased platelet production, two major types of thrombocytosis, i.e. platelet count 400×10^9/l can be found: reactive and clonal[3]. Reactive thrombocytosis (RT) is the commonest cause of an elevated platelet count and includes an heterogeneous group of disorders characterized by increased megakaryopoiesis produced by an intrinsecally normal megakaryocyte compartment. This is thought to be due to an increased cytokine production secondary to several pathological events (Table 41.1).

On the other hand, essential thrombocythemia (ET) and the thrombocythemic states found in the myeloproliferative disorders (MPDs) are usually characterized by a clonal thrombocytosis[4]. In this situation, a single hematopoietic precursor or stem cell and its progeny gain a growth advantage over their normal counterparts, so that the proliferation of the latter is suppressed. This growth advantage is derived, at least in part, from increased sensitivity of megakaryocytes and other hematopoietic precursors to the stimulatory effect of thrombopoietin (TPO), erythropoietin and several others growth factors[5,6]. The crucial role of TPO is supported by recent findings in families with hereditary thrombocythemia, where mutations in the TPO gene resulted in derepression of TPO mRNA translation, overproducton of TPO protein and thrombocytosis in the affected family members[7–9].

TPO is the primary regulator of megakaryocyte development and platelet number[10,11]. Produced predominantly by the liver and the kidney, the rate of TPO production is thought to be relatively constant, with no changes in mRNA levels or isoforms during thrombocytopenia or thrombocytosis[12]. TPO circulating levels are controlled by specific binding to its receptor, c-mpl, expressed on the surface of megakaryocytes and platelets, leading to its

internalization and degradation[13]. In thrombocytopenia due to hypoproductive states, as for example in aplastic anemia, blood TPO levels are inversely proportional to the platelet mass. In contrast, in thrombocytosis this relationship is not maintained. A number of studies have reported either normal or slighly elevated blood TPO levels in patients with both primary and secondary thrombocytosis[14,15] and no correlation between TPO levels and platelet count was demonstrated. Therefore, measurement of TPO cannot be used to discriminate between RT and ET or other MPDs. A possible explanation for elevated TPO levels in spite of elevated platelet count in MPDs has been suggested by the recent demonstration of reduced c-mpl expression on platelets from these patients[16]. Since c-mpl reduction was found restricted to clonal thrombocytosis, this finding has been proposed as a positive marker for these disorders[17,18], even though this remains to be confirmed in further studies. In spite of this new information, to date, the differential diagnosis between RT and ET still rests on the exclusion criteria discussed below[19].

Reactive thrombocytosis

In RT, the level of platelet counts is variable and may be very high. In a study of 280 cases with extreme thrombocytosis, i.e. platelet counts greater than or equal to 1000×10^9/l, 231 (82%) had RT and only 38 (14%) had a myeloproliferative disorder[20]. Characteristically, in this study, the proportion of patients with clonal thrombocytosis increased with age and a peak was reached in the highest age groups. In over 16000 platelet counts performed on 7916 children, it was found that 36 (0.5%) produced at least one count of $>800 \times 10^9$/l and seven (0.1%) had platelet counts $>1000 \times 10^9$/l. Most of them had an acute infection, although two were also recovering from antineoplastic chemotherapy and one was concomitantly iron deficient[21].

The great majority of patients with RT can be included in four groups, namely inflammatory disease, malignancy, splenectomy and iron deficiency anemia.

Inflammatory disease and malignancy

Thrombocytosis has long been recognized to accompany acute and chronic infections and also non-infectious inflammatory states. Early reviews stressed that thrombocytosis was commonly present during the course of bacterial pneumonia, pyelonephritis, osteomyelitis, pyogenic arthritis, chronic wound infections, and other bacterial infections[22]. Subsequently[23], 76% of patients with active pulmonary tuberculosis were reported to have elevated platelet counts, with 11% having platelet counts greater than 1000×10^9/l. In contrast, thrombocytosis is rare during the course of viral infections. The platelet count often falls to some degree along with the leukocyte count.

A similar degree of thrombocytosis accompanies a wide variety of noninfectious, inflammatory illnesses such as rheumatoid arthritis[24], vasculitis (including polyarteritis nodosa and polymyalgia rheumatica)[22], inflammatory bowel disease, hepatic cirrhosis[22], and nephritis[22]. In general, the degree of thrombocytosis parallels the activity of disease, and the elevated platelet count returns to normal levels with effective therapy.

An increase in platelet count is frequently seen in patients with advanced malignancy[22,25–27]. Ninety per cent of patients with thrombocytosis secondary to malignancy have platelet counts in the range of 400×10^9/l to 1000×10^9/l; occasional patients have been reported[25] with counts as high as 6000×10^9/l. The pathophysiologic mechanisms of the thrombocytosis of malignancy and inflammation may be similar. In a study of patients with carcinoma of the lung and colon[27], the platelet count correlated directly with the leukocyte count and the fibrinogen level and inversely with the hemoglobin, again suggesting that the platelet count may be regarded as an acute-phase reactant. Thrombocytosis has been reported with most types of tumours. The literature has stressed its frequency in carcinoma of the lung[27] and in mesothelioma[26].

Splenectomy

Approximately one-third of the total body platelet mass normally resides in a pool in the spleen. Therefore, the platelet count should theoretically rise by approximately 50% after splenectomy. The inflammation related to surgery might transiently increase the platelet count by approximately another 50%. However, if splenectomy is performed in a hematologically normal person, the platelet count often rises far in excess of that predicted by these two considerations, sometimes reaching in excess of 1000×10^9/l

Therefore, the removal of the spleen may stimulate platelet production. Thrombocytosis generally subsides over weeks to months and rarely persists for more than 2 years following splenectomy. Thus, the stimulation must be transient or compensated for in some circulating factor that suppresses platelet production, but this is not well established. It is generally accepted that such transient, self-limited postsplenectomy thrombocytosis is not associated with thrombotic or hemorrhagic complications[28] and that therapy for it is not necessary. Following splenec-

Cambridge Books Online © Cambridge University Press, 2009

tomy (even many years later), infectious or inflammatory thrombocytosis may be exaggerated considerably.

Postsplenectomy thrombocytosis can be expected to persist under two circumstances. A patient with MPD who has splenomegaly and a normal platelet count may, in fact, have a marked increase in the rate of platelet production which is masked by an increase in the size of the pool of platelets in the spleen. If the spleen is removed under these circumstances, there may be a massive increase in the platelet count, often to ten times the pre-splenectomy value. There are many reports of thrombotic complications in this situation[29]. The second circumstance occurs when the spleen is removed in patients with anemia secondary to hemolysis or ineffective erythropoiesis and the splenectomy does not completely correct the anemia[30]. Typical situations in which this might occur are hereditary, nonspherocytic hemolytic anemia and thalassemia major. In such patients, it is typical for the platelet count to persist indefinitely in the range of 600×10^9/l to 1000×10^9/l. In addition, such patients have an increased incidence of deep vein thrombosis[30].

Iron-deficiency anemia

Patients with anemia secondary to simple iron deficiency typically have an elevated platelet count, not uncommonly to levels in excess of 1000×10^9/l. In adults in the Western world, blood loss is the most common cause of iron deficiency, and bleeding itself can cause thrombocytosis. However, in experimental animals, iron deficiency produced by an iron-deficient diet also leads to thrombocytosis[31]. The mechanism is not known, and there is no explanation for the observation that some patients develop marked thrombocytosis while others with equally severe anemia have only modest elevations in platelet count. The platelet count falls rapidly as soon as iron replacement is begun, so that normal or even low levels may be reached within 7–10 days[32]. Thus the increased rate of platelet production appears to be secondary to iron deficiency *per se* and not to the erythroid hyperplasia in the marrow, which continues until the anemia is corrected. Although there have been case reports of thrombotic complications in this setting[32,33], the response of the thrombocytosis to iron replacement is so rapid that no other therapy is required.

Essential thrombocythemia

Within the category of clonal thrombocytosis, ET represents the most characteristic disease. Clonal hemopoiesis in ET was originally shown by studying glucose-6-phosphate deydrogenase (G6PD) isoenzymes in females who were coincidentally heterozygous at this C-linked locus[4]. More recent methods of DNA and RNA analysis have made it possible to assess clonality in the nucleated cells of up to 50% of women with these illness[34]. These analyses have confirmed the previous G6PD studies in most, but not in all patients. For example, 14 out of 46 patients (30%) studied by El-Kassar et al.[35] and 13 out of 46 patients (28%) studied by Harrison et al.[36] who had a diagnosis of ET according to the Polycytemia Vera Study Group (PVSG) criteria had polyclonal myelopoiesis. These results indicate that ET is a heterogeneous disease[37] and raise a number of issues, in particular whether etiology and clinical course in the two groups are different. It is possible that patients with monoclonal myelopoiesis represent those who have progressed from a polyclonal disease by acquiring a pathogenic alteration that provides cells with a growth or survival advantage[34]. This process may not necessarily reflect a truly ‘transformed’ state, since progression to acute leukemia is observed in only 6–7% of ET patients[19,38] and further hits may be required for transformation to occur. However, it should be recognized that there is no known experimental model to support such an hypothesis. From a clinical point of view, no difference between patients with monoclonal and polyclonal myelopoiesis was observed with respect to age or platelet count at diagnosis, incidence of hepatosplenomegaly or bleeding complications. However, monoclonal patients were more likely to have experienced thrombotic events[36,39]. This information is noteworthy and requires confirmation in larger and prospective studies since, as will be discussed in greater detail subsequently, the ability to categorize patients as potentially either ‘low risk’ or ‘high risk’ for thrombosis is the current basis for treatment recommendations.

Diagnostic criteria

There is no single clinical or laboratory finding that permits a positive diagnosis of ET. Rather, many clinicians have concluded that the diagnosis must be reached by excluding other myeloproliferative or myelodysplastic disorders or the conditions which are associated with a reactive thrombocytosis. This principle was used in the development of the criteria of the PVSG[40] that have been recently updated[19] (Table 41.2). A flow diagram for diagnosis of ET according to these criteria is presented in Fig. 41.1. The individual components will be discussed, including some investigations aimed at providing positive diagnostic tests for ET.

Cambridge Books Online © Cambridge University Press, 2009

Table 41.2. Updated diagnostic criteria for essential thrombocythemia[19]

I	Platelet count>600 000/μl
II	Hematocrit <40%, or normal RBC mass (Males <36 ml/kg, females <32 ml/kg)
III	Stainable iron in marrow or normal serum ferritin or normal RBC mean corpuscular volume[a]
IV	No Philadelphia chromosome or *bcr/abl* gene rearrangement
V	Collagen fibrosis of marrow A. Absent or B. <1/3 biopsy area without both marked splenomegaly and leukoerythroblastic reaction
VI	No cytogenetic or morphologic evidence for a myelodysplastic syndrome
VII	No cause for reactive thrombocytosis

Note:
[a] If these measurements suggest iron deficiency, PV cannot be excluded unless a trial of iron therapy fails to increase the RBC mass into the polycythemic range

Platelet count

The current criteria for ET give a persistent platelet count of 600×10^9/l[19]. However, it has been suggested that the platelet count criterion should be reduced to 400×10^9/l[41] since, in long-term follow-up studies, the clinical course of patients with platelet counts between 400 and 600×10^9/l was found to be indistinguishable from that of patients with a clearly diagnosed essential thrombocythemia[42,43]. The major problem with this approach is that platelet counts above these levels are commonly encountered in hospital practice and many individuals at the extreme end of the physiological range may be included. In other words, lowering the threshold of platelet count will improve the sensitivity but reduce the specificity of ET diagnosis. Therefore, the selection of a cut-off of 600×10^9/l is probably the most appropriate for the selection of ET patients to be put in therapeutic trials. In the current clinical practice, a lower platelet count can be taken for an initial screening but then a highly specific test, such as bone marrow histology (see below) should be suggested for establishing the correct diagnosis avoiding false-positive cases. In any case, these borderline patients deserve to be carefully followed up.

Platelet morphologic and functional abnormalities

ET platelets may be normal in size but more often their volume is highly variable with micro and giant platelets coexisting in the same patient[44,45]. The mean platelet volume (MPV) and size heterogeneity, reflected by the platelet distribution width (PDW), are easily measured by electronic cell counter, but their usefulness in the differential diagnosis with RT is questionable. The PDW is higher in ET than in RT but the overlap between groups makes it unreliable as the sole citerion[1]. The MPV is an unhelpful marker[46].

ET platelets are characterized by several biochemical and functional abnormalities[47]. Many defects are related to reductions of specific glycoproteins with impairment of the associated receptor functions[48,49]. Glycoprotein changes most commonly described are the reduction of GPIb[48] and GP IIb–IIIa complex[50] and/or modification of their glycosylation[51]. Binding sites for von Willebrand factor and fibrinogen are present on GPIb and GPIIb–IIIa, respectively, and this explains some loss of the binding capacity for these molecules[50]. In ET patients with extreme

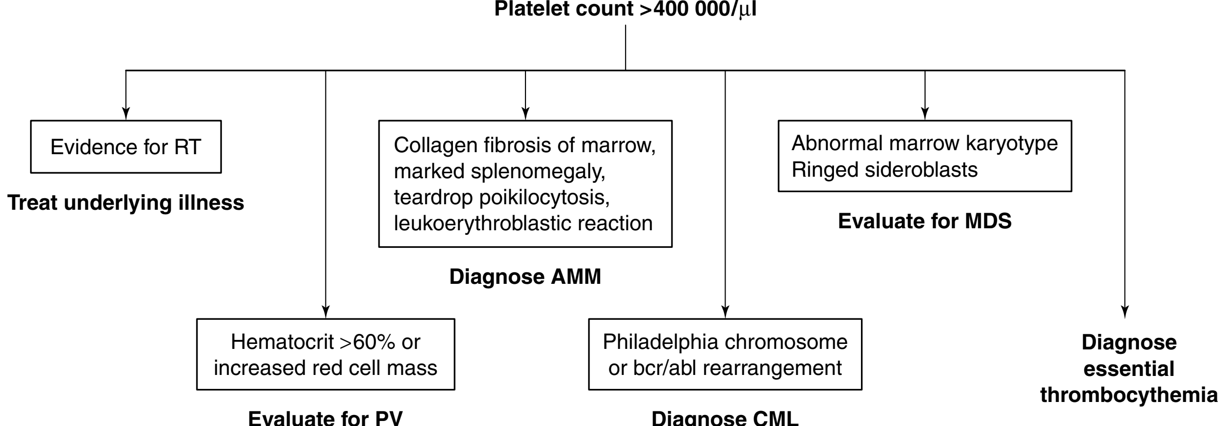


Fig. 41.1. Flow diagram for the diagnosis of essential thrombocythemia. See text for details. AMM, agnogenic myeloid metaplasia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; PV, polycythemia vera; RT, reactive thrombocytosis.

Cambridge Books Online © Cambridge University Press, 2009

thrombocytosis a plasma deficiency of von Willebrand large multimers has been described[52,53]. This deficiency, along with the decrease of platelet receptors for that factor, may explain the bleeding observed in thrombocythemic states (see also below).

Several alterations in arachidonic acid metabolism have been reported. These include reduced arachidonic acid content in platelet membranes[54] defective substrate mobilization in response to stimuli and abnormalities in its conversion through the PGH-synthase and 12-lipoxygenase pathways[55–57]. However, the relevance of these findings for diagnostic or prognostic purposes remains to be established.

Many functional studies on ET platelets have been performed by measuring platelet aggregation. The two most consistent findings refers to the loss of ET platelet aggregating response to epinephrine[58] and the phenomenon of spontaneous aggregation[59]. Intrinsic limitations of this technique have been reported, however[45], and its use in ET can be also associated with potential artefacts arising from variable ex vivo activation of ET platelets and modification of platelet morphology and size.

Other qualitative platelet changes include reduced ADP levels, leading to a higher ATP/ADP ratio in ET than in RT[46], and increased plasma levels of β-thromboglobulin[60,61] and platelet factor 4[62], suggesting enhanced platelet activation in vivo in ET subjects. However, again, these tests are of limited value because of the lack of standardization, limited availability and cost. Overall, despite the identification of a broad array of specific structural, biochemical and metabolic platelet defects, no parameter of hemostasis has been shown to reliably distinguish between ET and RT or herald a thrombotic or bleeding tendency in ET patients.

Exclusion of PV

If the patient's hematocrit is $>$51% for males or 48% for females the total body red cell mass should be measured. If it is elevated, the diagnosis should be PV[1,63]. If the red cell mass is not elevated, the possibility of PV, masked by bleeding or iron deficiency, should be considered. For practical purposes, a normal or increased serum ferritin level along with a normal red blood cell mean corpuscolar volume suffices to exclude both reactive thrombocytosis secondary to iron deficiency and the possibility that one may be dealing with PV masked by iron deficiency. When iron-deficiency erythropoiesis is present or cannot be excluded, the distinction between PV and ET cannot be made with certainty until re-valuation is performed after 1–2 months of iron replacement therapy.

Exclusion of other MPDs or myelodysplasia

During the initial evaluation of a patient suspected of having ET, it is advisable to perform a bone marrow aspiration, biopsy, and karyotype analysis[64–66]. If frank fibrosis on the bone marrow biopsy is found, the diagnosis of myelofibrosis can be made, particularly if other ancillary signs are present, such as marked splenomegaly on physical examination, teardrop poikilocytosis and a leukoerythroblastic reaction in the peripheral blood smear. The bone marrow karyotype is critical to exclude the Philadelphia (Ph) chromosome and, therefore, CML. In some circumstances in which the clinical picture is atypical for ET, the *bcr/abl* rearrangement, diagnostic of CML should be sought. Recently, an intriguing report suggested the presence of BCR-ABL transcripts in the bone marrow of a high proportion (48%) of Ph-neg ET patients[67]. However, this finding was not confirmed[68,69] and therefore its clinical significance of this finding remains to be determined. Sometimes, myelodysplastic syndromes (MDS) may present with thrombocytosis[70], particularly in the 5q- syndrome[71]. If present, this karyotypic abnormality would place the patient in the MDS category. However, it should be borne in mind that a proportion of patients with clinical and laboratory features overlapping between ET and other MPDs or MDS remains unclassified. Reliable prognostic information about such patients is not available and a careful follow-up to establish the clinical evolution is probably the most reasonable approach.

Exclusion of reactive thrombocytosis

The causes of RT are listed in Table 41.1 and discussed above. Clinical evaluation and laboratory studies designed to detect iron deficiency and occult inflammatory or malignant disease are generally sufficient to exclude RT. In addition, a study[72] suggested that most patients with RT have elevated serum levels of interleukin-6 and C-reactive protein while patients with MPD and thrombocytosis have normal levels.

Positive criteria for diagnosing ET

The approach to ET as a diagnosis of exclusion is intrinsically unsatisfactory, and positive criteria that would distinguish ET from RT and other MPDs have been proposed[46,73]. The role and limits of clonality studies and measurement of TPO and TPO receptor in this setting has been discussed above. Some authors have shown that endogenous erythroid colonies (EEC)[74] or culture examining CFU-Mk growth may be reliable markers of the disease[75,76]. However, these investigations are not widely available, are expensive and technically demanding and, therefore, may

Cambridge Books Online © Cambridge University Press, 2009

be suitable for the research purposes in the occasional patient but not for general use.

It has been suggested that ET can be positively diagnosed by careful, quantitative examination of the bone marrow biopsy[64–66]. Typical clustering of enlarged megakaryocytes with multilobated nuclei has been advocated to represent the hallmark feature of the disease. Histologic background of hematopoiesis in ET is featured by a discrete pattern of minimal or no prominence of erythropoiesis, no change in granulopoiesis, almost no fibrosis and reduction of stainable iron. A detailed evaluation of bone marrow features may also help to distinguish 'true' ET from the initial stages of idiopathic myelofibrosis[66] or myelodysplasia[65]. 'Early' myelofibrosis is characterized by increasing cellularity with prominent neutrophil granulopoiesis, borderline to slight reticulin fibrosis and pronounced abnormalities of megakaryocyte differentiation, including hyperchromasia and marked nuclear cytoplasmatic deviation. Notably, patients with these morphological features frequently develop an overt myelofibrosis and have a significantly worse life expectancy. However, an experienced observer and a well-standardized procedure are required to diagnose ET by examination of the bone marrow biopsy. This is one of the major limits of the diagnostic classifications of ET based on bone marrow histology.

Clinical features and prognostic factors

A diagnosis of ET, based on a careful pathologic and cytogenetic review, is associated with a very low risk of either leukemic transformation or occurrence of other life-threatening complications. As a result, life expectancy is near normal[77]. However, the clinical course of these patients is characterized by frequent thrombotic and hemorrhagic events. Thus, once the diagnosis is adequately established, the next step is to evaluate for the presence or absence of risk factors for thrombohemorrhagic complications.

Bleeding and thrombosis in ET

Reported rates for thrombosis and hemorrhage range from 8 to 84% and 13 to 63%, respectively[78–83]. This wide range reflects the referral bias in the reported series, which are almost all retrospective and based on relatively small numbers of patients. The vascular occlusive events either occur in the microvasculature, with symptoms such as transient cerebral ischemia, migraine and visual dysfunction[84] digital ischemia and erythromelalgia[85] or in larger vessels usually cerebral, peripheral and coronary arteries[45]. Major venous occlusions can also occur and thromboses in unusual sites such as hepatic (Budd–Chiari syndrome)[86,87], portal[87,88] and mesenteric veins[89] and of intracranial sinuses[90] are not infrequently encountered. Bleeding involves more frequently mucocutaneous sites rather than joints or soft tissues. Common manifestations are represented by easy bruising, epistaxis and gingival bleeding. Severe hemorrhages requiring transfusion and/or hospitalization are relatively infrequent and generally involve the gastrointestinal tract[45]. Due to this heterogenous clinical presentation, several studies have focused on the search for factors possibly associated with an increased risk of thrombotic and hemorrhagic complications[78–83].

Risk factors for major bleeding

Hemorrhagic symptoms have been found more frequently in patients with platelet counts in excess of 1000×10^9/l and this may be related to an acquired deficiency of vonWillebrand's factor[52,53]. The number of circulating platelets directly affects the concentration of plasma large vWF multimers, which may compromise hemostasis at high platelet counts. In a series of 44 ET patients under the age of 45, 6 (13%) experienced bleeding complications which for the most part were mild and mucosal in nature. In two cases, however, deep-seated hemorrhage occurred into muscle and one of these individuals was found to have an acquired von Willebrand's disease[91]. In these patients, serious bleeding may be spontaneous or triggered by contemporaneous aspirin treatment[92]. Therefore, caution is recommended in giving platelet inhibiting agents to ET patients with platelet counts above 1000×10^9/l. Bleeding time measurement performed by the Ivy's method have been widely used in ET patients in order to identify patients with impaired platelet function and increased hemorrhagic risk[93]. However, there is no established evidence that bleeding time has any predictive value for the evaluation of the hemorrhagic risk associated with surgical procedures[94], antiplatelet therapy or in patients with congenital platelet defects[95–96]. Thus, bleeding time measurement seems of uncertain clinical usefulness in ET patients[47].

Risk factors for thrombosis

A clear correlation between ageing and thrombosis has been reported by many authors[80,97–100]. In a cohort study of 100 consecutive patients with ET diagnosed between 1978 and 1988, the rate of thrombosis was 1.7% per year in patients younger than 40, versus 6.3% per year in those from 40–60 years and 15.1% per year in subjects over 60 years old[80]. In another series of 56 young thrombocythemic subjects the incidence of severe thromboses was only about 1% per year after a mean follow-up of 4.7 years[97].

Cambridge Books Online © Cambridge University Press, 2009

However, isolated reports indicate the possibility of life-threatening thromboses also in young ET patients[91,101–104]. Most ischemic complications in this group were represented by migraine, headache and erythromelalgia. The thrombotic nature of these neurological symptoms is uncertain, however. In a study of transient neurological and ocular ischemias in patients with ET, Michiels et al. reported that poorly localized symptoms such as dysarthria, transient unsteadiness, and scintillating scotomata were more common than focal symptoms such as transient monocular blindness or transient mono- or hemi paresis[105]. These clinical manifestations were uniformly sensitive to aspirin (see also below) and often preceded or followed by erytromelalgia or large vessel thrombosis.

A previous history of thrombotic events is an important risk factor for subsequent vascular occlusions, despite the fact that most symptomatic patients are treated with cytoreductive drugs. In one study, the prevalence of thrombosis was increased by the presence of a prior thrombotic event from 3.4% per year to 31.4% per year[80] and in another, from 16 to 57%[83].

Disease transformation

In rare cases of ET, transformation to polycythemia vera or myelofibrosis may occur as part of the natural history of disease. This is consistent with the well-known overlap between different Philadelphia-negative CMDs. In a long-term study of young women with ET, transformation into polycythemia vera and myelofibrosis was observed in 2.7% and 4% of the patients, respectively[106]. Sporadic reports of the transformation of ET to acute leukemia have been recorded, although the incidence appears less than in the related MPDs. Retrospective studies with median duration follow-up of 3 to 7 years have reported incidence rates of leukemic conversion ranging from 0.6 to 5%[79,82,83,107,108]. Most patients who had transformation were previously managed with cytoreductive therapy (see below). However, leukemic transformation in ET has occasionally occurred in the absence of previous therapy, suggesting that the event may also be a natural, albeit very rare, sequela of the disease.

Table 41.3. Risk stratification in essential thrombocythemia

Low-risk
Age <60 years, and
No history of thrombosis, and
Platelet count $<1500\times10^9$/l
High-risk
Age 60 years, or
A previous history of thrombosis, or
Platelet count 1500×10^9/l

Note:
Correction of cardiovascular risk factors (smoking, obesity) is recommended in all patients.

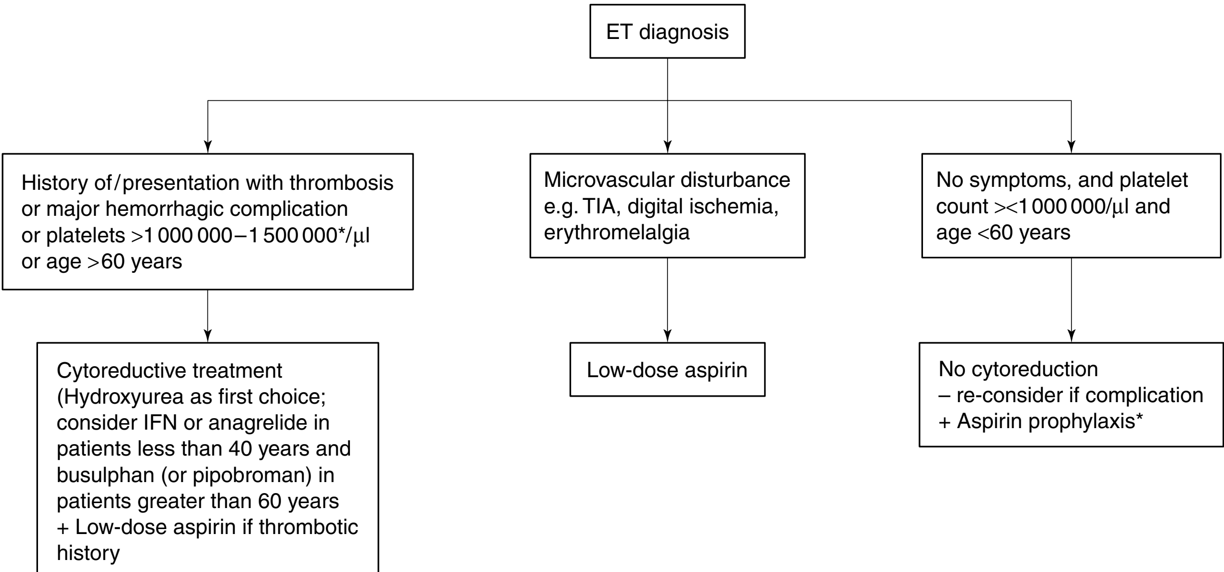


Fig. 41.2. Flowchart of recommended treatment for patients with essential thrombocythemia. Areas of uncertainty are marked with the asterisk (*). IFN, interferon; TIA, transient ischemic attack.

Cambridge Books Online © Cambridge University Press, 2009

Treatment

The management of patients with ET is based on a careful evaluation for the presence or absence of risk factors for thrombohemorrhagic complication and should take into consideration the issue of potential leukemogenicy associated with myelosuppressive drugs (Table 41.3 and Fig. 41.2).

Management of 'lower-risk' patients

The natural history of untreated, 'lower-risk' ET patients was evaluated in a prospective, controlled study[109]. Sixty-five ET patients with age below 60 years, no history of thrombosis or major bleeding and platelet count below 1500×10^9/l were compared to 65 age- and sex-matched normal controls. Patients were not treated with cytoreductive therapy, until the occurrence of major clinical events. After a median follow-up of 4.1 years, the incidence of thrombosis in patients and controls was 1.91 and 1.5% patient–year, respectively. The age and sex adjusted risk rate ratio was 1.43 (95% c.i. 0.37–5.4). No major bleeding was observed. This study indicates that the thrombotic risk of young, asymptomatic ET patients is not significantly increased compared to the normal population. These findings have been recently confirmed in another Italian cohort of 28 patients below 40 years of age followed for a median of 4 years[100]. However, it should be recognized that the number of patients included in these studies is relatively small and further data from large, currently ongoing clinical trials are warranted. Thrombotic deaths have been only occasionally reported in young patients with ET[91] and data showing that fatalities can be prevented by starting cytoreductive drugs early have not been produced. Therefore, one can conclude that, to date, withholding cytoreductive therapy might be justifiable in asymptomatic, young ET patients with a platelet count below 1000 $\times 10^9$/l, based on their very low risk of developing fatal thrombotic or bleeding events and for the concern about the potential leukemogenicity of cytotoxic drugs (see below).

Having made this generalization concerning myelosuppressive therapy, it may be wise to propose a primary antithrombotic prophylaxis with aspirin for reducing the rate of vascular complications in these asymptomatic patients. Aspirin was found to be effective in ET patients with microvascular disturbances, such as erythromelalgia[85] or transient cerebral or ocular ischemia[84]. In the absence of contraindications and/or side effects, a dose of 100–300 mg/day is recommended in the acute management of these patients, and 75–100 mg/day are also recommended as secondary prophylaxis for major arterial thrombosis[45]. However, in the absence of clinical trials in the specific setting of ET, this recommendation is largely based on the assumption that the use of platelet antiaggregating agents is associated with a risk reduction comparable to that achievable in patients not having ET. In prescribing antiaggregating agents one should consider the risk of bleeding, an issue particularly important in ET patients, who very often have platelet functional defects and a possible abnormal hypersensitivity to aspirin[47,92].

Management of 'high-risk' patients

Patients aged more than 60 years or with platelet count exceeding 1500×10^9/l, or with a previous history of thrombosis or major bleeding are candidates to receive a cytoreductive drug[106,110].

Hydroxyurea

Hydroxyurea (HU) has emerged as the treatment of choice in patients with ET because of its efficacy and only rare acute toxicity[111]. The drug is given at an initial dose of 15–20 mg/kg/day, with adjustments to maintain reduced platelet values, ideally to less than 400×10^9/l without excessive lowering of the neuthrophil count. Treatment with HU has been shown to reduce the platelet count to below 500×10^9/l within 8 weeks in 80% of patients. Hematopoietic impairment, leading to neutropenia and macrocytic anaemia, is the major short-term toxic effect of HU. Neutropenia is dose related and generally quickly reversible if the drug is discontinued for a few days. Withdrawal is followed by a rebound of platelet counts, so that continuous treatment is necessary. Failure of HU to provide adequate control of the platelet count was reported in 11% to 17% of patients[19,112]. Additional side effects of the drug may include skin changes[113,114], nail staining[115] and leg ulcerations[114,116]. In occasional patients, these dermatological symptoms may be severe enough to withdraw the drug.

Lowering the platelet count by HU is associated with significant improvement in acute ischemic or hemorrhagic symptoms. In a randomized clinical trial, the efficacy of HU in preventing thrombosis in ET patients aged >60 years, or with a history of previous thrombosis, or with a platelet count above 1500×10^9/l was clearly demonstrated[117].

Is hydroxyurea leukemogenic?

HU, being a non-alkylating agent, was generally thought to be non-mutagenic, but long-term follow-up studies of HU-treated patients with PV and ET revealed that some cases developed acute leukemia[118–120]. Even when used as a single agent in the treatment of PV and ET, the reported

Cambridge Books Online © Cambridge University Press, 2009

rate of acute leukemia ranges between 3.5 and 10% and this fatal complication is encountered 4–10 years after the start of treatment[121]. In other clinical conditions, the use of HU was rarely associated with secondary malignancies. However, it should be mentioned that at least three patients with sickle cell disease treated with HU developed leukemia, after 6–8 years of treatment[122].

The incidence of leukemic transformation is enhanced in patients with ET carrying cytogenetic abnormalities[123]. About 5% of ET patients have alterations, mostly involving chromosomes 1, 2, 5, 17, 20 and 21. The 17p deletion has been recently described in a high proportion of ET patients who developed acute myeloid leukemia and myelodysplasia following treatment with hydroxyurea[120].

The importance of avoiding multiple cytotoxic drugs with different mechanisms of action (antimetabolites, alkylating agents) in ET patients has been emphasized[19,38,124]. In a follow-up study of the unique randomized clinical trial hitherto performed, 56 high-risk ET patients randomized to receive HU and 58 to receive no cytoreductive therapy have been observed for a median observation period of 73 months (range 3–94). Before randomization, 15 patients had been treated with busulphan. When analyzed by intention to treat, seven patients (13%) in the HU group developed secondary acute leukemia, myelodysplastic syndromes or solid tumours, but only one control (1.7%) ($P = 0.032$). The occurrence of secondary malignancies was also analysed by treatment: none of 20 patients never treated with chemotherapy developed neoplasia vs. 3 of 77 given HU only (3.9% n.s.) and 5 of 15 given busulfan plus HU (33% $P < 0.0001$). Thus, the sequential use of busulfan and HU significantly increased the risk of second malignancies[38]. These findings are consistent with those of other studies. Sterkers et al. reported 14% rate of leukemia when HU was combined with other cytotoxic agents, generally pipobroman[120]. Six cases of AML (21%) out of 28 ET patients treated with HU plus alkylating agents or 32P were observed by Murphy et al[19].

The reason why sequential cytotoxic therapy is associated with an high rate of second malignancies is uncertain. One reason could be that an inadequate disease control with the first drug selects a subset of patients with a resistent disease and an increased tendency to develop leukemia. Murphy et al[19]. suggested another possible explanation, that is more consistent with our data. The first myelosuppressive drug can act as a ‘sensitizer’ such that exposure to it followed by a ‘second hit’ is highly leukemogenic. Supporting this hypothesis, Sterkers et al[120]. observed that any combination of cytotoxic agents was associated with an increased rate of second leukemia.

In conclusion, the definite proof that HU is leukemogenic is still lacking. However, there is increasing evidence that the drug may accelerate the progression to acute leukemia in predisposed patients, such as those with cytogenetic abnormalities[120] or given multiple cytotoxic drugs[38].

Busulphan

Busulphan is an alkylating agent interfering with the megakaryocyte proliferation[125]. It should be given in a dose of 2–4 mg daily, according to hematological response, under weekly control of platelet count. After normalization of the platelet count, adequate long-term control of thrombocytosis can be obtained with intermittent courses of the drug, allowing long intervals without the need for therapy. With this schedule, the secondary effects of the drug usually observed at higher doses, such as bone marrow aplasia, skin pigmentation, amenorrhea and pulmonary fibrosis, can be avoided[125,126]. Alkylating agents, such as chlorambucil, were associated with an excess incidence of malignancies in polycythemia vera[127]. Although busulphan was not found to induce leukemia or other cancers in PV and ET patients[125,126,128], concerns about leukemogenicity suggest to limit its indication to elderly patients, where the convenience of its use outweigh the potential leukemogenic risk.

Interferon

Recombinant interferon-α (IFN) is an active agent in myeloproliferative disorders with cytoreductive activity that is virtually devoid of mutagenic risk and is being used both in ET and PV[129]. The rationale for this drug includes its myelosuppressive activity and its ability to antagonize the action of platelet-derived growth-factor (PDGF), a product of megakaryopoiesis which initiates fibroblast proliferation. The precise mechanism of action, however, has not yet been fully elucidated. In ET patients, IFN has been evaluated in several cohort studies[130]. Overall results indicate that reduction of platelet count below 600×10^9/l can be obtained in about 90% of cases after about 3 months with an average dose of 3 million IU daily. Time and degree of the platelet reduction during the induction phase were dose dependent. During maintenance the IFN dose could be tapered, but if IFN is suspended platelet count rebounds in the majority of patients. IFN is not known to be teratogenic and it does not cross the placenta. Thus, it has been used successfuly throughout pregnancy in some ET patients with no adverse fetal or maternal outcome.

Side effects are a major problem with this drug[130–132]. Fever and flu-like symptoms are experienced by most patients and usually require contemporaneous administration of paracetamol. Signs of chronic IFN toxicity, such as weakness, myalgia, weight and hair loss, severe depres-

Cambridge Books Online © Cambridge University Press, 2009

sion and gastrointestinal and cardiovascular symptoms, necessitate drug cessation in a relevant proportion of patients. In 273 cases published in the literature[130], IFN therapy was terminated in 25% (67 cases) against the primary treatment plan. The rate of withdrawal ranged between zero and 66% in the different studies. The wide range may be partly explained by the difference in the observation time lasting from 1 month to 4 years in the reported patients. The most common reasons for withdrawal were IFN-related side effects in 55% and patient refusal in 10%. Thus far, no leukemogenic effects have been reported. Therefore, despite high cost and toxicity, IFN remains a promising agent in cytoreductive treatment of ET, especially in younger patients.

A new interesting development is represented by the pegylated form of IFN[133]. This recent formulation may be one step forward because of its slower clearance, permitting once-weekly dosing. Clinical studies comparing IFN (also in its pegylated form) to HU or anagrelide are currently ongoing.

Anagrelide

Anagrelide is a member of the imidazo(2,1-b)quinazolin-2-one series of compounds with an inhibitory activity on platelet aggregation in both humans and animals. In addition, it has in humans a species-specific platelet-lowering effect observed at dose levels lower than those required to inhibit platelet aggregation. Because of this, the drug has been tested in patients with clonal thrombocytosis and has been shown to have potent platelet reducing activity[131,134]. The mechanism whereby anagrelide reduces platelet count without affecting the white count or normally the red blood cells is not yet completely understood, but there are data showing that its major action is the inhibition of megakaryocytic maturation. No chromosomal damage has been reported in relation to its use. The efficacy of anagrelide in ET has been assessed in non-comparative clinical studies[134]. Response was defined as a platelet count <500 or 600×10^9/l or a 50% drop in platelet count. A response rate of 60 to 93% was reported, irrespective of age, sex, spleen size, bleeding time, clinical symptoms or prior treatment. The average dose required to control platelet count was 2 to 2.5 mg per day and, in most cases, the median time for response was 3–4 weeks, although delayed responses of up to 24 months were described. Patients refractory to HU responded to anagrelide in 68% of cases. Continuous therapy is required because the platelet count rebounds in a few days after withdrawal of the drug.

The most serious complications of anagrelide are cardiac, including palpitations or forceful heart beats (27% of patients) tachycardia and other arrhythmias ($<10\%$) and congestive heart failure (2%)[131]. In addition, the vasodilating effect of the drug is the underlying cause of headache, which is the more frequent side effect (occurring in more than one-third of patients), fluid retention or edema (24% of cases), dizzness (15%) and postural hypotension. Gastrointestinal complications (nausea, abdominal pain and diarrhea) and transient rash have been reported less frequently. Sudden death was observed in two patients given anagrelide: one death was related to a pulmonary infiltrate and the other to congestive heart failure. Overall, 16% of 424 evaluable thrombocythemic patients with MPDs, including 262 with ET, discontinued anagrelide treatment because of side effects. Leukemic transformation has not been observed for up to 55 months of treatment.

The long-term efficacy and safety of Anagrelide has been recently analysed in 35 young patients with ET (median age 38 yrs, range 17–48) followed in a single Institution for a median follow-up period of 10.8 years (range 7–15.5)[135]. The overall initial response rate was 94% and the reduction of platelet count was maintained in 66% of patients over the study period. Eight patients (24%) experience a more than 3 g/dl decrease in hemoglobin level and three (9%) discontinued treatment because of toxicity. Most importantly, 7 patients (20%) experienced a total of 10 thrombotic episodes, while on therapy, and a similar proportion experienced major bleeding complications. None of the patients developed acute leukemia. Thus, long-term therapy with anagrelide appears to be relatively well tolerated but thrombohemorrhagic complications continue to occur. The final place of this drug in the therapeutic strategy of ET patients remains to be established in controlled clinical trials, such as the PT1 trial currently ongoing in UK and Germany.

Special situations (pregnancy, surgery, children)

Some special clinical situations in ET patients deserve consideration. Approximately 50% of pregnancies develop obstetric complications, including recurrent abortion, particularly in the first trimester, premature delivery, fetal growth retardation, and abruptio placenta[136]. The likely mechanism of these complications is placental infarction, so that some authors recommend the use of aspirin[137] or subcutaneous heparin[138], particularly in individuals who have had previous fetal losses. By reviewing the relevant literature, it would appear that aspirin was associated with a more successful outcome of pregnancy than in those managed without the drug[137]. Thus, aspirin, 75 mg/day, throughout the pregnancy and for at least 6 weeks after delivery is recommended in all women enrolled in a prospective, follow-up study currently ongoing in UK (T.C.

Cambridge Books Online © Cambridge University Press, 2009

Pearson, personal communication). However, in a single institutional experience from Mayo Clinic[139], specific therapy during 34 pregnancies did not appear to modify the clinical outcome. In symptomatic patients, the use of α-interferon has been proposed[140] even though only very few anecdotal reports support this recommendation. Thus, some uncertainty still remains in the management of pregnant patients with ET and we recommend considering therapy on an individual basis.

Considering major surgery, patients with uncontrolled PV and ET have an increased risk of thrombosis and hemorrhage in the postoperative period. Based on this rationale, a course of myelosuppressive therapy to lower the platelet count of patients with MPD should be considered prior to elective surgery. Myelosuppressive therapy is particularly indicated in patients with myeloproliferative disorders who require splenectomy, since such patients may have severe thrombo-haemorrhagic complications associated with massive postsplenectomy thrombocytosis.

ET may be observed in children[141–144]. These very young patients are generally characterized by a persistently elevated platelet count above 900×10^9/l and frequent headache, thrombosis and hemorrhagic episodes often leading to the discovery of the disease. There is some uncertainty to consider these thrombocytoses as 'true' myeloproliferative diseases and the term 'idiopathic thrombocytosis' has been suggested to emphasize this point. A careful long-term follow-up of all these cases is recommended to answer this question and establish the natural history of ET beginning in childhood.

Thrombocytosis in polycythemia vera and other MPDs

Increased platelet count is a frequent finding in PV and is currently included among the criteria for diagnosis[145]. Whether thrombocytosis should be considered as a risk factor for thrombosis is uncertain, however[146]. In a nested case-control analysis performed in the setting of the PVSG-01 protocol[147], each patient who suffered a thrombosis was matched with a thrombosis-free control of similar age, sex, treatment group and duration of study. Platelet counts at the nearest time before the thrombotic events of the index cases were not significantly different from those of the matched controls, suggesting that the level of the platelet count itself is not responsible for thrombosis.

Yet many clinicians believe that lowering of the platelet count in patients with PV leads to a reduction in thrombotic complications. This is supported by the finding that myelosuppressive therapy is associated with fewer thrombotic complications than therapy with phlebotomy alone[127]. However, one must ask whether myelosuppressive therapy reduces the incidence of thrombosis because of reduction of platelet count or whether thrombosis results from some other aspect of PV which is yet ill defined. In this regard, a recent study showed that in ET and PV an in vivo activation of the polymorphonuclear leukocytes occurs and is associated with laboratory signs of endothelium and coagulation system activation[148]. Nevertheless, there is no information, to date, on the effect of myelosuppressive therapy on this pathophysiologic mechanism.

Another important issue relates to the potential profibrotic effect of uncontrolled thrombocytosis in ET and other MPDs, driven by platelet-derived growth factor (PDGF) and other cytokines produced by platelets and megakaryocytes. In a French trial comparing hydroxyurea and pipobroman for the treatment of PV, a significant increase in the risk of progression to myelofibrosis was seen in the HU-treated patients[149]. Three-quarters of the patients developing myelofibrosis had permanently raised platelet counts ($> 400 \times 10^9$/l), particularly in the HU arm.

In idiopathic myelofibrosis, elevated platelet count is frequently observed after splenectomy, when major thrombotic and bleeding complications are more likely to occur[111]. In these cases, the risk of perioperative thrombosis may be reduced by judicious prophylactic use of drugs that lower the platelet count[150].

References

1 Pearson TC. Diagnosis and classification of erythrocytosis and thrombocytosis. In Green AR, Pearson TC, eds. *Myeloproliferative disorders.* London: Bailliere's Clinical Haematology; 1998; 11: 695–720.
2 Williams WJ. Secondary thrombocytosis. In Williams WJ, Beutler E, Lichtman MA, Coller BS, Kipps TJ eds. *Hematology*, 5th edn. New York: McGraw-Hill; 1995: 1361–1363.
3 Barbui T, Finazzi G. Thrombocytosis. In Loscalzo J, Schafer AI, eds. *Thrombosis and haemorrhage*, Baltimore: Williams and Wilkins; 1998: 665–682.
4 Fialkow PJ, Faguet GB, Jacobson RJ et al. Evidence that essential thrombocythemia is a clonal disorder with origin in a multipotent stem cell. *Blood* 1981; 58: 916–919.
5 Prchal JT, Prchal JF. Evolving understanding of the cellular defect in polycythemia vera: implications for its clinical diagnosis and molecular pathophysiology. *Blood* 1994; 83: 1–4.
6 Green AR. Pathogenesis of polycythemia vera. *Lancet* 1996; 347: 844–845.

Cambridge Books Online © Cambridge University Press, 2009

7 Wiestner A, Schlemper RJ, van der Maas et al. An activating splice donor mutation in the thrombopoietin gene causes hereditary thrombocythemia. *Nat Genet* 1998; 18: 49.
8 Kondo T, Okabe M, Sanada M et al. Familial essential thrombocythemia associated with one-base deletion in the 5′-untranslated region of the thrombopoietin gene. *Blood* 1998; 92: 1091–1096.
9 Ghilardi N, Skoda RC. A single-base deletion in the thrombopoietin (TPO) gene causes familial essential thrombopoietin through a mechanism of more efficient translation of TPO mRNA. *Blood* 1999; 94: 1480–1482.
10 Levin J. Thrombopoietin – clinically realized? *N Engl J Med* 1997; 336: 434–436.
11 Kaushansky K. Regulation of megakaryopoiesis. In *Thrombosis and haemorrhage*, Loscalzo J and Schafer AI, eds. Baltimore: Williams and Wilkins; 1998: 173–194.
12 Cohen-Solal K, Villeval J-L, Titeux M et al. Constitutive expression of Mpl ligand transcripts during thrombocytopenia or thrombocytosis. *Blood* 1996; 88: 2578.
13 Fielder PJ, Gurney AL, Stefanich E et al. Regulation of thrombopoietin levels by c-mpl-mediated binding to platelets. *Blood* 1996; 87: 2154.
14 Cerutti A, Custodi P, Duranti M et al. Thrombopoietin levels in patients with primary and reactive thrombocytosis. *Br J Haematol* 1997; 99: 281.
15 Pitcher L, Taylor K, Nichol J et al. Thrombopoietin measurement in thrombocytosis: dysregulation and lack of feedback inhibition in essential thrombocythemia. *Br J Haematol* 1997; 99: 929.
16 Horikawa Y, Matsumura I, Hashimoto K et al. Markedly reduced expression of platelet c-mpl receptor in essential thrombocythemia. *Blood* 1997; 90: 4031.
17 Moliterno AR, Silver RT, Spivak J. A diagnostic assay for polycythemia vera. *Blood* 2000; 96: 512a.
18 Mesa RA, Li CY, Yoon SY et al. Clinical correlates of megakaryocyte c-mpl expression in essential thrombocythemia: a clinico-pathological study of 169 patients. *Blood* 2000; 96: 742a.
19 Murphy S, Peterson P, Iland H et al. Experience of the Polycythemia Vera Study Group with essential thrombocythemia: a final report on diagnostic criteria, survival and leukemic transition by treatment. *Semin Hematol* 1997; 34: 29–39.
20 Buss DH, Cashell AW, O'Connor ML et al. Occurrence, etiology, and clinical significance of extreme thrombocytosis: a study of 280 cases. *Am J Med* 1994; 96: 247–253.
21 Vora AJ, Lilleyman JS. Secondary thrombocytosis. *Arch Dis Child* 1993; 68: 88–90.
22 Selroos O. Thrombocytosis. *Acta Med Scand* 1973; 193: 431–436.
23 Baynes RD, Bowthwell TH, Flax H et al. Reactive thrombocytosis in pulmonary tuberculosis. *J Clin Path* 1987; 40: 676–679.
24 Ghosh P. Thrombocytosis in rheumatoid arthritis. *Br J Clin Pract* 1986; 40: 388–389.
25 Spiegel SC, Mooney LR. Extreme thrombocytosis associated with malignancy. *Cancer* 1977; 39: 339–341.
26 Chahinian AP, Pajak TF, Holland JF et al. Diffuse malignant mesothelioma. *Ann Intern Med* 1982; 96: 746–755.
27 Costantini V, Zacharski LR, Moritz TE et al. The platelet count in carcinoma of the lung and colon. *Thromb Haemost* 1990; 64: 501–505.
28 Boxer MA, Braun J, Ellman L. Thromboembolic risk of postsplenectomy thrombocytosis. *Arch Surg* 1978; 113: 808–809.
29 Gordon DH, Schaffner D, Bennet JM et al. Postsplenectomy thrombocytosis. *Arch Surg* 1978; 113: 713–715.
30 Hirsh J, Dacie JV. Persistent post-splenectomy thrombocytosis and thrombo-embolism: a consequence of continuing anaemia. *Br J Haematol* 1966; 12: 44–53.
31 Schloesser LL, Kipp MA, Wenzel FJ. Thrombocytosis in iron-deficiency anemia. *J Lab Clin Med* 1965; 66: 107–114.
32 Knizley H, Noyes WD. Iron deficiency anemia, papilledema, thrombocytosis and transient hemiparesis. *Arch Intern Med* 1972; 129: 483–486.
33 Heller Ds, Pervez NK, Kleinerman J. Fatal cerebrovascular thrombosis in a young woman: an unusual complication associated with hypocromic anemia and thrombocytosis following surgery. *Mt Sinai J Med* 1988; 55: 318–20.
34 Gale RE. Biological heterogeneity in essential thrombocythemia and its clinical implications. *Hematology*, ASH Education Program Book 1999; 178–184.
35 El-Kassar N, Hetet G, Briere J et al. Clonality analysis of hematopoiesis in essential thrombocythemia: advantages of studying T lymphocytes and platelets. *Blood* 1997; 89: 128.
36 Harrison CN, Gale RE Machin SJ et al. A large proportion of patients with a diagnosis of essential thrombocythemia do not have a clonal disorder and may be at lower risk of thrombotic complications. *Blood* 1999; 93: 417.
37 Nimer SD. Essential thrombocythemia: another 'heterogeneous disease' better understood? *Blood* 1999; 93: 415–416.
38 Finazzi G, Ruggeri M, Rodeghiero F et al. Second malignancies in patients with essential thrombocythemia treated with busulphan and hydroxyurea: long-term follow-up of a randomized clincal trial. *Br J Haematol* 2000; 110; 577–583.
39 Chiusolo P, Sica S, Ortu La Barbera E et al. Patients with essential thrombocythemia and clonal hemopoiesis detected by X-inactivation pattern are at high risk for thrombotic complication. *Blood* 1999; 94 (Suppl. 1): 700a.
40 Murphy S, Iland H, Rosenthal D et al. Essential thrombocythemia: an interim report from the polycythemia very study group. *Semin Hematol* 1986; 23: 177–182.
41 Michiels JJ, Juvonen E. Proposal for revised diagnostic criteria of essential thrombocythemia and polycythemia vera by the Thrombocythemia Vera Study Group. *Semin Thromb Haemost* 1997; 23: 339–347.
42 Lengfelder E, Hochhaus A, Kronawitter U et al. Should a platelet limit of 600×10^9/l be used as a diagnostic criterion in essential thrombocythemia? An analysis of the natural course including early stages. *Br J Haematol* 1998; 100: 15–23.
43 Sacchi S, Vinci G, Gugliotta L et al. Diagnosis of essential

Cambridge Books Online © Cambridge University Press, 2009

thrombocythemia at platelet counts between 400 and 600 × 10^9/l. *Haematologica* 2000; 85: 492–495.

44 Thompson CB, Jakubowsky JA. The pathophysiology and clinical relevance of platelet heterogeneity. *Blood* 1988; 72: 1–8.

45 Landolfi R, Rocca B, Patrono C. Bleeding and thrombosis in myeloproliferative disorders: mechanisms and treatment. *Crit Rev Oncol Hematol* 1995; 20: 203–222.

46 Dudley JM, Messinezy M, Eridani S et al. Primary thrombocythaemia: diagnostic criteria and a simple scoring system for positive diagnosis. *Br J Haematol* 1989; 71: 331–335.

47 Finazzi G, Budde U, Michiels JJ. Bleeding time and platelet function in essential thrombocythemia and other myeloproliferative syndromes. *Leuk Lymphoma* 1996; 22 (suppl.1): 71–78.

48 Bolin RB, Okumura T, Jamieson GA. Changes in distribution of platelet membrans glycoproteins in patients with myeloproliferative disorders. *Am J Hematol* 1977; 3, 63–71.

49 Eche N, Caranobe C, Nouvel C, Pris J, Boneu B. Platelets in myeloproliferative disorders III. Glycoprotein profile in relation to platelet function and platelet density. *Scand J Haematol* 1981; 26: 123–129.

50 Mazzucato M, De Marco L, De Angelis V, Deroia D, Bizzaro N, Casonato A Platelet membrane abnormalities in myeloproliferative disorders – decrease in glycoprotein-IB and glycoprotein-IIb/IIIa-complex is associated with deficient receptor function. *Br J Haematol* 1989; 73: 369–374.

51 Clezardin P, McGregor PL, Dechevanne M, Clementson MC. Platelet membrane glycoprotein abnormalities in patients with myeloproliferative disorders and secondary thrombocytosis. *Br J Haematol* 1985; 60: 331–344.

52 Budde U, Scharf RE, Franke P et al. Elevated platelet count as a cause of abnormal von Willebrand factor multimer distribution in plasma. *Blood* 1993; 82: 1749–1757.

53 Van Genderen PJJ, Michiels JJ, van der Poel-van de Luytgaarde SCPAM et al. Acquired von Willebrand disease as a cause of recurrent mucocutaneous bleeding in primary thrombocythemia: relationship with platelet count. *Ann Hematol* 1994; 69: 81–84.

54 Keenan J, Wharton J, Shepherd A et al. Defective platelet lipid peroxidation in myeloproliferative disorders: a possible defect of prostaglandin synthesis. *Br J Haematol* 1977; 85: 275–283.

55 Okuma M, Uchino H. Altered arachidonate metabolism by platelets in patients with myeloproliferative disorders. *Blood* 1979; 54: 1258–1271.

56 Schafer AI. Deficiency of platelet lipoxygenase activity in myeloproliferative disorders. *N Engl J Med* 1982; 306: 381–386.

57 Cortelazzo S, Galli M, Castagna D, Viero P, De Gaetano G, Barbui T. Increased response to arachidonic acid and U-46619 in patients with chronic myeloproliferative disorders. *Thromb Haemost* 1988; 59: 73–76.

58 Kaywin P, McDonough M, Insel PA, Shattil S. Platelet function in essential thrombocythemia: decreased epinephrine responsiveness associated with a deficiency of platelet alpha-adrenergic receptors. *N Engl J Med* 1978; 299: 505–509.

59 Wu KK. Platelet hyperaggregability and thrombosis in patients with thrombocythemia. *Ann Intern Med* 1978; 88, 7–11.

60 Cortelazzo S, Viero P, Barbui T. Platelet activation in myeloproliferative disorders. *Thromb Haemost* 1981; 45: 211–213.

61 Viero P, Cortelazzo S, Bassan R, Barbui T. Effect of aspirin on platelet 5-hydroxytryptamine and beta-thromboglobulin plasma levels in patients with myeloproliferative diseases. *Thromb Haemost* 1982; 48: 125–126.

62 Burstein SA, Malpass TW, Yee et al. Platelet factor-4 in myeloproliferative disease: implications for the etiology of myelofibrosis. *Br J Haematol* 1977; 57: 383–392.

63 Pearson TC, Guthrie DL, Simpson J et al. Red cell mass and plasma volume interpretation in normal adults. *Br J Haematol* 1995; 89: 748–756.

64 Georgii A, Buesche G, Kreft A. The histopathology of chronic myeloproliferative diseases. In *Myeloproliferative disorders*. Green AR, Pearson TC eds. London: Baillière's Clinical Haematology 1998; 11: 721–750.

65 Annaloro C, Lambertenghi-Deliliers G, Oriani A et al. Prognostic significance of bone marrow biopsy in essential thrombocythemia. *Haematologica* 1999; 84: 17–21.

66 Thiele J, Kvaniscka HM, Zankovich R et al. Relevance of bone marrow features in the differential diagnosis between essential thrombocythemia and early stage idiopathic myelofibrosis. *Haematologica* 2000; 85: 1126–1134.

67 Blickstein D, Aviram A, Luboshitz M et al. BCR–ABL transcripts in bone marrow aspirates of Philadelphia-negative essential thrombocythemia patents: clinical presentation. *Blood* 1997; 190: 2768–2771.

68 Marasca R, Luppi M, Zucchini P et al. Might essential thrombocythemia carry Ph anomaly? *Blood* 1998; 91: 3084–3085.

69 Rambaldi A, Finazzi G, Dotti GP et al. Lack of BCR-ABL transcripts in bone marrow aspirates of essential thrombocythemia patients. *Haematologica* 1999; 84 (EHA-4 Abstr. Book): 118.

70 Jondeau K, Bouscary D, Viguie F et al. Thrombocythemia and abnormal megakaryopoiesis associated with abnormality of chromosome 1p34 in myelodysplastic syndrome. *Leukemia* 1996; 10: 1692–1695.

71 Boultwood J, Lewis S, Wainscoat JS. The 5q- syndrome. *Blood* 1994; 84; 3253–3260.

72 Tefferi A, Ho TC, Ahmann GJ et al. Plasma interleukin-6 and C-reactive protein levels in reactive versus clonal thrombocytosis. *Am J Med* 1994; 97: 374–378.

73 Kutti J, Wadenvik H. Diagnostic and differential criteria of essential thrombocythemia and reactive thrombocytosis. *Leuk Lymph* 1996; 22 (Suppl. 1): 41–46.

74 Shih LY, Lee CT. Identification of masked polycythemia vera from patients with idiopathic marked thrombocytosis by endogenous erythroid colony assay. *Blood* 1994; 83: 744–748.

75 Juvonen E, Ikkala E, Oksanen K, Ruutu T. Megakaryocyte and erythroid colony formation in essential thrombocythemia and reactive thrombocytosis: diagnostic value and correlation to complications. *Br J Haematol* 1993; 83: 192–197.

Cambridge Books Online © Cambridge University Press, 2009

76 Rolovic Z, Basara N, Gotic M et al. The determination of spontaneous colony formation is an unequivocal test for discriminating between essential thrombocythemia and reactive thrombocytosis. *Br J Haematol* 1995; 90: 326–331.
77 Rozman C, Giralt M, Feliu U et al. Life expectancy of patients with chronic nonleukemic myeloproliferative disorders. *Cancer* 1991; 67: 2658–2663.
78 Barbui T, Cortelazzo S, Viero P et al. Thrombohaemorrhagic complications in 101 cases of myeloproliferative disorders: Relationship to platelet number and function. *Eur J Cancer Clin Oncol* 1983; 19: 1593–1599.
79 Bellucci S, Janvier M, Tobelem G et al. Essential thrombocythemia: Clinical evolutionary and biological data. *Cancer* 1986; 58: 2440–2447.
80 Cortelazzo S, Viero P, Finazzi G et al. Incidence and risk factors for thrombotic complications in a historical cohort of 100 patients with essential thrombocythemia. *J Clin Oncol* 1990; 8: 556–562.
81 Hehlmann R, Jahn M, Baumann B et al. Essential thrombocythemia: Clinical characteristics and course of 61 cases. *Cancer* 1988; 61: 2487–2496.
82 Fenaux P, Simon M, Caulier MT et al. Clinical course of essential thrombocythemia in 147 cases. *Cancer* 1990; 66: 549–556.
83 Colombi M, Radaelli F, Zocchi L et al. Thrombotic and hemorrhagic complications in essential thrombocythemia. *Cancer* 1991; 67: 2926–2930.
84 Koudstaal PJ, Koudstaal A. Neurologic and visual symptoms in essential thrombocythemia: efficacy of low-dose aspirin. *Semin Thromb Haemost* 1997; 23: 365–370.
85 Van Genderen PJJ, Michiels JJ. Erythromelalgia: a patognomonic microvascular thrombotic complication in essential thrombocythemia and polycythemia vera. *Semin Thromb Haemost* 1997; 23 (4): 357–364.
86 Valla D, Casadevall N Lacombe C et al. Primary myeloproliferative disorder and hepatic vein thrombosis. *Ann Intern Med* 1985; 103: 329–334.
87 De Stefano V, Teofili L, Leone G et al. Spontaneous erythroid colony formation as the clue to an underlying myeloproliferative disorder in patients with Budd–Chiari syndrome or portal vein thrombosis. *Semin Thromb Haemost* 1997; 23: 411–418.
88 Valla D, Casadevall N, Huisse MG et al. Myeloproliferative disorders in portal vein thrombosis in adults. *Gastroenterology* 1988; 94: 1063–1069.
89 Tossou H, Iglicki F, Casadevall N et al. Superior mesenteric vein thrombosis as a manifestation of a latent myeloproliferative disorder. *J Clin Gastroenterol* 1991; 13: 597–598.
90 Haan J, Caekebeke JFK, Van der Meer FJM et al. Cerebral venous thrombosis as presenting sign of myeloproliferative disorders. *J Neurol Neurosurg Psychiat* 1988; 5: 1219–1220.
91 Mitus AJ, Barbui T, Shulman LN et al. Hemostatic complications in young patients with essential thrombocythemia. *Am J Med* 1990; 88: 371–375.
92 Barbui T, Buelli M, Cortelazzo S et al. Aspirin and risk of bleeding in patients with thrombocythemia. *Am J Med* 1987; 83: 265–268.
93 Schafer AI. Bleeding and thrombosis in myeloproliferative disorders. *Blood* 1984; 64: 1–12.
94 Lind SE. The bleeding time does not predict surgical bleeding. *Blood* 1991; 77: 2547–2552.
95 Channing Rodgers RP, Levin J. A critical reappraisal of the bleeding time. *Semin Thromb Haemost* 1990; 16: 1–20.
96 De Caterina R, Lanza M, Manca G, Buti Strata G, Maffei S, Salvatore L. Bleeding time and bleeding: an analysis of the relationship of the bleeding time test with parameters of surgical bleeding. *Blood* 1994; 84: 3363–3370.
97 McIntyre K, Hoagland H, Silverstein M et al. Essential thrombocythemia in young adults. *Mayo Clin Proc* 1991; 66: 149–154.
98 Randi ML, Fabris F, Rossi C et al. Sex and age as prognostic factors in essential thrombocythemia. *Haematologica* 1992; 77: 402–404.
99 Besses C, Cervantes F, Pereira A et al. Major vascular complications in essential thrombocythemia: a study of the predictive factors in a series of 148 patients. *Leukemia* 1999; 13: 150–154.
100 Randi ML, Luzzatto G, Fabris F. Low-risk thrombocythemia in patients younger than 40. *Br J Haematol* 1999; 104: 928–936.
101 Davis RB. Acute thrombotic complications of myeloproliferative disorders in young adults. *Am J Clin Pathol* 1985; 84: 180–185.
102 Decoulx E, Millaire A, Jude B et al. Arterial thromboses and essential thrombocythemia in young patients. *Ann Cardiol Angiol* 1990; 39: 347–350.
103 Randi ML. Multiple, relapsing thrombosis in a young man with primary thrombocytosis. *Blood Coagul Fibrinolysis* 1990; 1: 331–332.
104 Randi ML, Rossi C, Fabris F et al. Essential thrombocythemia in young adults: major thrombotic complications and complications during pregnancy – a follow-up study in 68 patients. *Clin Appl Thromb Haemost* 2000; 6: 31–35.
105 Michiels J, Koudstaal P, Mulder A et al. Transient neurological and ocular manifestation in primary thrombocythemia. *Neurology* 1993; 43: 1107–1110.
106 Tefferi A. Risk-based management in essential thrombocytemia. *Hematology; ASH education program book* 1999; 172–177.
107 Chistolini A, Mazzucconi MG, Ferrari A et al. Essential thrombocythemia: a retrospective study on the clinical course of 100 patients. *Haematologica* 1990; 75: 537.
108 Cervantes F, Tassies D, Salgado C et al. Acute transformation in nonleukemic chronic myeloproliferative disorders: actuarial probability and main characteristics in a series of 218 patients. *Acta Haematol* 1991; 85: 124.
109 Ruggeri M, Finazzi G, Tosetto A et al. No treatment for low-risk essential thrombocythemia: results from a prospective study. *Br J Haematol* 1998; 103: 772–777.
110 Barbui T, Finazzi G. Management of essential thrombocythemia. *Crit Rev Oncol/Hematol* 1999; 29: 257–266.
111 Barbui T, Finazzi G and Barosi G. Chronic myeloid disorders, excluding CML. In Degos L, Linch DC, L'wenberg B, eds. *Textbook of malignant haematology*, London: Martin Dunitz 1999; 847–870.

Cambridge Books Online © Cambridge University Press, 2009

112 Barbui T, Finazzi G, Dupuy E et al. Treatment strategies in essential thrombocythemia. *Leuk Lymphoma* 1996; 22 (Suppl.1): 149–160.
113 Kennedy BJ, Smith LR, Goltz RW. Skin changes secondary to hydroxyurea therapy. *Arch Dermatol* 1975; 111: 183–187.
114 Kirby B, Gibson LE, Rogers S et al. Dermatomyositis-like eruption and leg ulceration caused by hydroxyurea in a patient with psoriasis. *Clin Exp Dermatol* 2000; 25: 256–257.
115 Pirard C, Michaux JJ, Bourlond A. Longitudinal melanonychia and hydroxyurea. *Ann Dermatol Venereol* 1994; 121: 106–109.
116 Disla E, D'Eamour L, Cioriou M. Hydroxyurea-associated leg ulceration. *Ann Intern Med* 1998; 129: 252–253.
117 Cortelazzo S, Finazzi G, Ruggeri M et al. Hydroxyurea in the treatment of patients with essential thrombocythemia at high risk of thrombosis: a prospective randomized trial. *N Engl J Med* 1995; 332: 1132–1136.
118 Weinfield A, Swolin B, Westin J. Acute leukaemia after hydroxyurea therapy in polycythemia vera and allied disorders: prospective study of efficacy and leukaemogenicity with therapeutic implications. *Eur J Haematol* 1994; 52: 134–139.
119 Nand S, Stock W, Godwin J et al. Leukemogenic risk of hydroxyurea therapy in polycythemia vera, essential thrombocythemia and myeloid metaplasia with myelofibrosis. *Am J Hematol* 1996; 52: 42–46.
120 Sterkers Y, Preudhomme C, Lai J-L et al. Acute myeloid leukemia and myelodysplastic syndromes following essential thrombocythemia treated with hydroxyurea: high proportion of cases with 17p deletion. *Blood* 1998; 91: 616–622.
121 Finazzi G, Barbui T. Treatment of essential thrombocythemia with special emphasis on leukemogenic risk. *Ann Hematol* 1999; 78: 389–392.
122 Steinberg MH. Current use of hydroxyurea in sickle cell disease. *Hematology; ASH education program book* 2000; 9–17.
123 Lifvenberg E, Nordenson I, Walhlin A. Cytogenetic abnormalities and leukemic transformation in hydroxyurea-treated patients with Philadelphia chromosome negative chronic myeloproliferative disease. *Cancer Genet Cytogenet* 1990; 49: 57–67.
124 Randi ML, Fabris F, Girolami A. Leukemia and myelodysplasia: effect of multiple cytotoxic therapy in essential thrombocythemia. *Leuk Lymph* 2000; 37: 379–385.
125 Van de Pette JEW, Prochazka AV, Pearson TC et al. Primary thrombocythemia treated with busulfan. *Br J Haematol* 1986; 62: 229–237.
126 Berberi A, Shvidel L, Shtalrid et al. Short course of busulphan in essential thrombocythemia: remodelling of an old strategy. *Br J Haematol* 2000; 109: 249–250.
127 Berk P, Goldberg J, Donovan P et al. Therapeutic recommendations in polycythemia vera based on polycythemia vera study group protocols. *Semin Hematol* 1986; 23: 132–143.
128 Haanen C, Mathe G for the EORTC. Treatment of polycythaemia vera by radioactive phosphorus or busulphan. *Br J Cancer* 1981; 44: 75–80.
129 Silver RT. Recombinant interferon alpha for treatment of polycythemia vera: *Lancet* 1988; ii: 403.
130 Lengfelder E, Griesshammer M, Hehlmann R. Interferon-alpha in the treatment of essential thrombocythemia. *Leuk Lymphoma* 1996; 22 (Suppl.1): 135–142.
131 Tefferi A, Elliot MA, Solberg LA Jr et al. New drugs in essential thrombocythemia and polycythemia vera. *Blood Rev* 1997; 11: 1–7.
132 Lengfelder E, Berger U, Hehlmann R. Interferon-alpha in the treatment of polycythemia vera. *Ann Hematol* 2000; 70: 103–109.
133 Talpaz M, O Brien S, Cortes J et al. Phase I study of pegylated interferon a-2A (Pegasys™) in patients with chronic myelogenous leukemia (CML). *Blood* 1999; 94 (Suppl.1): 530a.
134 Anagrelide Study Group. Anagrelide, a therapy for thrombocythemic states: experience in 577 patients. *Am J Med* 1992; 92: 69–76.
135 Storen EC, Tefferi A. Long-term use of anagrelide in young patients with essential thrombocythemia. *Blood* 2001; 97: 863–866.
136 Griesshammer M, Bergmann L, Pearson TC. Fertility, pregnancy and the management of myeloproliferative disorders. In *Myeloproliferative disorders*. Green AR, Pearson TC eds. London, Baillière's Clinical Haematology 1998; 11: 859–874.
137 Griesshammer M, Heimpel H, Pearson TC. Essential thrombocythaemia and pregnancy. *Leuk Lymphoma* 1996; 22 (suppl.1): 57–63.
138 Pagliaro P, Arrigoni L, Muggiasca ML et al. Primary thrombocythemia and pregnancy. Treatment and outcome in fifteen cases. *Am J Hematol* 1996; 53: 6–10.
139 Beressi AH, Tefferi A, Silverstein MN, Petitt RM, Hoagland CH. Outcome analysis of 34 pregnancies in women with essential thrombocythemia. *Arch Intern Med* 1995; 155: 1217–1222.
140 Shpilberg O, Shimon I, Sofer O et al. Transient normal platelet count and decreased requirement for interferon during pregnancy in essential thrombocythemia. *Br J Haematol* 1996; 92: 491–493.
141 Michiels JJ, Van Genderen PJJ. Essential thrombocythemia in childhood. *Semin Thromb Haemost* 1997; 23: 295–301.
142 Randi ML, Putti MC, Fabris F et al. Features of essential thrombocythemia in childhood: a study of five children. *Br J Haematol* 2000; 108: 86–89.
143 Chan GCF, Ma SK, Ha SY et al. Childhood essential thrombocythemia without evidence of myeloproliferation: how many investigations should be done? *Br J Haematol* 2000; 110: 1002.
144 Yang RC, Qian LG. Essential thrombocythemia in children: a report of nine cases. *Br J Haematol* 2000; 110: 1009–1010.
145 Pearson TC, Messinezy M, Westwood AR. Evaluation of current diagnostic pathways in the erythrocytoses. *Hematology, ASH education program book* 2000; 51–68.
146 Barbui T, Finazzi G The basis of current management and future perspectives in polycythemia vera. *Hematology, ASH education program book*; 2000: 61–68.
147 Murphy S. Megakaryocytes, platelets and coagulation in the myeloproliferative diseases. In *Polycythemia vera and the myeloproliferative disorders*, Wasserman LR, Berk PD, Berlin NI eds. Philadelphia W.B. Saunders; 1995: 102–113.

Cambridge Books Online © Cambridge University Press, 2009

148 Falanga A, Marchetti M, Evangelista V et al. Polymorphonuclear leukocyte activation and hemostasis in patients with essential thrombocythemia and polycythemia vera. *Blood* 2000; 96: 4261–4266.
149 Najean Y, Rain JD for the French Polycythemia Study Group. Treatment of polycythemia vera: the use of hydroxyurea and pipobroman in 292 patients under the age of 65 years. *Blood* 1997; 90: 3370–3377.
150 Tefferi A. Myelofibrosis with myeloid metaplasia. *N Engl J Med* 2000; 342: 1255–1265.

Cambridge Books Online © Cambridge University Press, 2009

42

Platelet adhesive protein defect disorders

Kenneth J. Clemetson and Jeannine M. Clemetson

Theodor Kocher Institute, University of Berne, Freiestrasse 1, CH-3012 Berne, Switzerland

Introduction

The investigation of platelet defects causing bleeding disorders has been an important step in determining the roles of the major glycoproteins in platelet function. In particular, Bernard–Soulier syndrome and Glanzmann's thrombasthenia have provided the basis for studying the function of the GPIb complex and $\alpha_{IIb}\beta_3$, respectively. More recently, some generally milder bleeding disorders have been related to the absence or deficiency of other platelet receptors. With the development of techniques to produce mice in which specific gene expression has been deleted, these defects can also be reproduced and can be corrected by gene therapy. Have the disorders in humans still lessons to teach us? Probably the main diseases have already been discovered but we cannot exclude that a detailed screening of patients with mild, platelet related, bleeding disorders might disclose defects in previously unknown minor platelet receptors or new mutations with different effects in known receptors. Many patients with mild bleeding problems related to platelets are seen in hematology departments and cannot yet be diagnosed at a molecular level. Achieving the ability to diagnose the defects in these patients is still a major goal in platelet studies and may be amenable to proteomics approaches. Until X-ray crystallographic structures are available for the GPIb complex and $\alpha_{IIb}\beta_3$, information provided by the molecular diagnosis of Bernard–Soulier syndrome and Glanzmann's thrombasthenia will continue to provide important data on structure–functional relationships.

GPIb-V–IX complex

Bernard–Soulier syndrome

Bernard–Soulier syndrome (BSS) is caused by genetic defects in the genes of GPIbα, GPIbβ or GPIX[1–3]. First reported in 1948[4] the molecular diagnosis of this disorder began in the 1970s[5,6] with the discovery of the major platelet glycoprotein GPIb, later shown to consist of two subunits[7]. The involvement of two other components, GPIX and GPV, in BSS and also in the GPIb complex, was demonstrated in the early 1980s[8,9]. Absence or defects in the fourth component of the complex, GPV[10], do not lead to BSS[11] and expression of GPV is not essential for the expression of the other three subunits although expression of GPV depends on the expression of the other subunits. Recent studies with GPV-null mice indicate that a defect in GPV might rather lead to a thrombotic disorder than a bleeding disorder[12,13]. Patients with BSS have a prolonged bleeding time, large, often giant, platelets and thrombocytopenia[14,15]. In fact, because of the size of the platelets, the total platelet mass may be normal. Platelet counts as well as platelet size vary considerably between different cases. The characteristic platelet defect in BSS is lack of response to ristocetin/von Willebrand factor, which is not corrected by addition of normal plasma, while responses to other agonists, with the exception of low doses of thrombin, are normal. The inheritance of BSS is normally autosomal recessive and when homozygous is often due to consanguinity, only one case has been described as autosomal dominant[16]. Typical symptoms include nose bleeds, bleeding from the gums and from minor scratches as well as a noticeable tendency to bruise easily. Severe, life-threatening, bleeding is associated with surgery or with major trauma and is normally controlled by platelet or

blood transfusions. In the past BSS was often misdiagnosed as idiopathic thrombocytopenia purpura and treated by splenectomy or steroids. Most older patients have undergone a splenectomy. Women with BSS require special attention because of menorrhagia and childbirth. Associated problems may be prevented by oral contraceptives and appropriate transfusions, respectively. Nowadays, most patients, in taking adequate precautions, can live relatively normal lives. Members of patients' families who are heterozygous for BSS normally show few, if any, symptoms. Occasionally, they have been reported to have larger than normal platelets or to show some mild signs such as easy bruising[17] but not all heterozygotes have these and it is not clear if these symptoms are only apparent because of a combination with a further partial defect, e.g. in a coagulation factor (however, see the A156V mutation, below).

In addition to their large size and lack of binding to vWf, platelets from patients with BSS show some further characteristics; they have a reduced response to low doses of thrombin[18] and they have abnormal procoagulant responses[19]. On the one hand, they have an increased resting procoagulant state and on the other they do not show a normal increase in procoagulant activity after stimulation. These phenomena have been at least partly explained by a recent series of studies showing a critical role for GPIbα in the platelet response to thrombin in general[20,21] and in the procoagulant response in particular[22]. The reason for the large platelets is still not satisfactorily explained. An obvious possibility that has been proposed several times is a reduced association between the membrane and the submembranous cytoskeleton which might also explain the increased membrane deformability found in BSS platelets[23].

The basic types of BSS are less varied than in Glanzmann's thrombasthenia (GT) because most molecular defects lead to lack of expression of the complex rather than to expression of a dysfunctional complex. Identified mutations or deletions affecting GPIbα are listed in Table 42.1 and those affecting GPIbβ and GPIX in Table 42.2. Molecular defects causing BSS can be classed as:

(i) Glycoproteins totally absent – caused by frame shifts, deletions and folding defects in any of the three chains GPIbα, GPIbβ, or IX followed by a failure to form a complex or of the complex to translocate to the membrane. Major categories within this type are caused by frame shifts or deletions in any of the three chains leading to premature termination and therefore lack of the transmembrane domain and production of soluble fragments. Typical examples in GPIbα are: deletion of T in codon 76 leading to frame shift and premature truncation[24]; W343stop[25]; S444stop[26]; W498stop[27]; one base deletion leading to frame shift and premature truncation[28]; one base deletion in codon 19 leading to frame shift and premature truncation at codon21[29]; two base deletion at 972–975 of gene leading to frame shift after T294 and premature termination[30]; base insertion at 1418, and deletion at 1438–1444 both leading to frame shift and premature termination[30]; deletion of two bases of codon 492 producing a truncated molecule detectable in plasma[31]. In GPIX these are W126stop[28,32] and in GPIbβ a base deletion in A80 resulting in a frame shift and premature termination[33] as well as a W21stop mutation[34].

(ii) Variants of these may be mutations in or near the transmembrane region leading to frame shifts within this domain or in the cytoplasmic domain. These can lead to partial expression of functional or dysfunctional molecules. Folding problems can also lead to failure to translocate to the membrane. Such problems are often caused by mutations leading to replacement of cysteine residues or to the introduction of abnormal cysteine residues (Fig. 42.1). These types of mutations have been seen in all three subunits, for example in GPIbα C209S[35], in GPIX C8R[36] C73Y[37], C97Y[38] and in GPIbβ Y88C[39]. Folding problems are also typically caused by mutations within the leucine-rich repeat (LRR) domains (Fig. 42.1). Examples in GPIbα are L129P[40], in GPIX L40P[41], N45S[17,42], F55S[43] and in GPIbβ N63T[44]. This last mutation in GPIbβ is particularly significant in confirming the presence of two LRR in this subunit (and most likely in GPIX also) as predicted by protein modelling, and not one LRR with 'flanking domains' which would not fold stably. The N45S mutation is particularly common in populations of northern European origin, e.g.[45] and has also been found in many unpublished cases.

(iii) There are a few mutations in the LRR domains that appear to affect folding less radically and result in partial expression of a molecule, which does not function normally. The cases so far described of these variants were all mutations of GPIbα: L57F[16], A156V[46], L179 deletion[47]. The A156V mutation[48] is particularly common in southern Italian populations and, like L57F, shows autosomal dominant inheritance characteristics. It should be noted that most of the mutations leading to single amino acid substitutions in the LRR domains causing BSS are in the β-sheet part of the repeat (see Fig. 42.1). An exception to this is F55S in GPIX. Presumably the α-helix is more forgiving of single amino acid substitutions. A few other cases of BSS are known where the mutations lie outside these domains or defects. These include mutations within the disulfide loops at each end of the LRR, again presumably causing problems by preventing the loops from folding correctly. In GPIbα mutations in the C-terminal loops can lead to

Cambridge Books Online © Cambridge University Press, 2009

Table 42.1. Mutations in GPIbα leading to Bernard–Soulier syndrome or pseudo-von Willebrand disease

Exon	Mutation	Phenotype	AA substitution	Genotype	Patient	Ref
Bernard–Soulier syndrome						
1	103delA	Del: out of frame	Premature termination	Homozygote	GA	29
1	165–168del	Del: out of frame	Premature termination	Compound heterozygote	Houston	31
1	217C>T	Missense	L57F	Heterozygote	NY Family	16
1	241T>C	Missense	C65R	Compound heterozygote	Iceland	113
1	275delT	Del: out of frame	Premature termination	Homozygote	Amsterdam	24
1	434T>C	Missense	L129P	Homozygote	TH, AJ	40
1	515C>T	Missense	A156V	Homozygote? Heterozygote	Bolzano	46,114
1	554–589del	Del: in frame	169–180del	Compound heterozygote	Naples	115
1	583–585del	Del: In frame	L179del	Homozygote	Nancy-1	116
1	673T>A	Missense	C209S	Homozygote	ML	35
1	930–931del	Del: out of frame	Premature termination	Homozygote	Fukuoka	30
1	1077G>T	Nonsense	W343X	Compound heterozygote	La Jolla	25
1	1379C>A	Nonsense	S444X	Homozygote	Kagoshima	117
1	1376insT	Ins: out of frame	Premature termination	Compound heterozygote	Fukuoka	30
1	1396–1402delA	Del: out of frame	Premature termination	Homozygote	Fukuoka	28,30
1	1523–1524del	Del: out of frame	Premature termination	Homozygote	Texas	118
1	1542G>A	Nonsense	W498X	Compound heterozygote	Iceland	27,113
Pseudo- (or Platelet-type) von Willebrand's disease						
1	746G>T	Missense	G233V	Heterozygote	Syracuse AR,TR,MaR	51
1	763A>T	Missense	M239V	Heterozygote	NagoyaPT	52,119

Table 42.2. Mutations in GPIbβ and GPIX leading to Bernard–Soulier syndrome

Exon	Mutation	Phenotype	AA substitution	Genotype	Patient	Ref
GPIbβ						
2	133>G GATA Site	No transcript		Compound heterozygote	DiGeorgePA	50
2	296C>G	Missense	P74R	Homozygote	HaT	49
2	338A>G	Missense	Y88C	Compound heterozygote	AK	39
2	397G>C	Missense	A108P	Compound heterozygote	AK	39
GPIX						
2	70T>C	Missense	C8R	Homozygote	Pakistan/MD	36
2	110A>G	Missense	D21G	Compound heterozygote	II-3,II-5,II-6	42
2	167T>C	Missense	L40P	Homozygote	Pavia-II	41
2	182A>G	Missense	N45S	Compound heterozygote	II-3,II-5,II-6	42
				Homozygote	WK, Finnish	17,45
2	212T>C	Missense	F55S	Homozygote	Pavia-I	43
2	266G>A	Missense	C73Y	Homozygote	HiroshimaIX	37
2	338G>A	Missense	C97Y	Homozygote	Nagoya-II	38
2	426G>A	Nonsense	W126X	Homozygote	Nagoya-I	120

Cambridge Books Online © Cambridge University Press, 2009

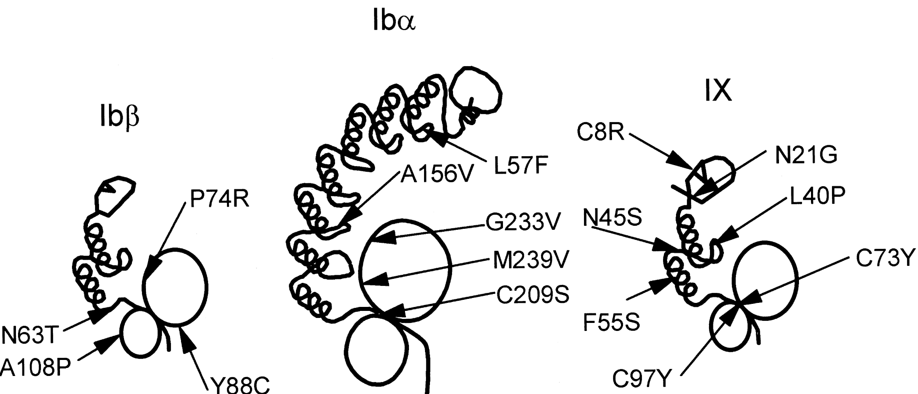


Fig. 42.1. Diagram of the outer, ligand-binding domains of GPIbα, GPIbβ and GPIX, consisting of leucine-rich repeats and disulfide bond linked loops, and indicating the position of amino acid substitution mutations involved in Bernard–Soulier syndrome and platelet-type von Willebrand disease.

platelet-type von Willebrand disease (see below). In GPIX mutations are found in N21G[42] and in GPIbβ in P74R[49], Y88C and A108P[39]. These last two mutations were characterised by the lack of disulfide bond formation between GPIbα and GPIbβ in most of the GPIb molecules. No functionally related mutations have so far been described within the macroglycopeptide region of GPIbα or in the region between the LRR C-terminal disulphide loops and the membrane surface in GPIbβ or GPIX.

(iv) All of the mutations listed above cause the disorder by inheritance in a recessive mode. The only example described as being dominant was a L57F mutation present in one allele of GPIbα[16]. More recently, the A156V mutation has been shown to have some autosomal dominant inheritance characteristics for a mild to moderate bleeding problem and larger than normal platelets as well as thrombocytopenia[48]. Since the other allele is normal in these patients, the phenotype could only be explained if a defective, expressed GPIbα interferes with the function of the normal molecule. No satisfactory mechanism to explain this has been suggested yet.

(v) Mutations within the promoter region may also affect expression levels leading to BSS. A typical example is a mutation in the GATA-1 sequence of the GPIbβ promoter in one allele which, combined with deletion of the other allele in DiGeorge syndrome, reduced expression of the complex to produce BSS[50].

Although still a rare disease and less common than GT, BSS is probably at least 2–3 times more common (2–3 cases per 1 000 000 population) than once thought. One reason for this is that recurrent mutations are not sufficiently novel to be published in the literature and are therefore underreported. An approach to solving this problem will be to provide a registry as has been done for GT (see below) to be able to collect information on all patients.

Platelet-type (or pseudo) von Willebrand's disease

Platelet-type von Willebrand's disease (vWd) is a dominant inherited bleeding disorder caused by mutations in GPIbα leading to an increased avidity for vWf in the resting platelet. This leads to platelet activation and aggregation, removal from the circulation by the spleen and periodic thrombocytopenia. Because the most active, larger multimers of vWf are preferentially removed, this causes defective hemostasis and hence bleeding risk. So far, two naturally occurring mutations, G233V[51] and M239V[52,53], leading to this disorder have been found (Table 42.1), both lying within the complex double-loop region between the LRR domain and the macroglycopeptide domain (Fig. 42.1). In vitro mutagenesis and expression of recombinant molecules have indicated that other mutations within this domain could also lead to changes in resting avidity[54] so that it remains possible that some of these mutations will be detected in vivo in the future. Nevertheless, it must be stressed that this is an extremely rare disorder probably because of the life threatening bleeding which is associated with it, which may well have been an evolutionary handicap in the past. Platelet-type vWd is generally treated very conservatively. Some cases may have been diagnosed as idiopathic thrombocytopenia purpura or as Type IIB vWd and it is important to differentiate these.

Cambridge Books Online © Cambridge University Press, 2009

Size polymorphisms in GPIbα and their relation to bleeding disorders

At least three patients are known who have mild bleeding disorders related to size variation in GPIbα[55–57]. All three of these showed two bands for GPIb on polyacrylamide gels with an abnormally high upper band compared to the known size polymorphisms. However, only one of these patients has been investigated in any detail. Polymorphisms in the size of GPIbα are due to different repeats of a 13-amino acid sequence present in the macroglycopeptide domain[58]. The commonest of these are the single copy or the duplicate but triple and quadruple repeats are also known from European and East Asian populations[59], respectively. More recently, a variant where this repeat is completely missing has also been described[60]. In the patient with the bleeding problem one of the alleles for GPIbα is normal whereas the other has six copies of the 13 amino acid repeat. The disorder is inherited in a dominant pattern and appears to be linked to the size polymorphism. It is not clear why this leads to a bleeding problem since the GPIb binding sites should stick out more from the membrane. Possible reasons might be the discordance in size between the normal and abnormal GPIb molecules affecting efficient binding to vWf. There have been several studies examining a possible role of size polymorphisms in GPIbα in cardiovascular disease[61–64] but no clear tendency has emerged unlike the situation with other platelet receptors.

$\alpha_{IIb}\beta_3/\alpha_v\beta_3$

Glanzmann's thrombasthenia

Glanzmann's thrombasthenia (GT) is at the same time the commonest and among the most complex of disorders caused by defects in platelet adhesive receptors. It was the first such bleeding disorder to be described in 1918[65] and a molecular explanation had to wait until 1974–5 when the glycoproteins called GPIIb–IIIa were first implicated[66,67]. It is now clear that this disorder is caused by a defect or lack of expression in these two glycoproteins forming the integrin $\alpha_{IIb}\beta_3$. This disorder is characterized by a lack or reduction in platelet aggregation to all agonists. A further typical symptom is the lack of fibrinogen in the platelet alpha granules in the extreme form of this disease. As in BSS the problem can be caused by mutations or deletions, which prevent expression of one or the other of the subunits by premature termination before the transmembrane region but there are also a great many variants with different levels of expression caused by mutations which also affect folding[15,68]. Both homozygous as well as compound heterozygous mutations have been reported. Unlike BSS, because of the larger number of introns/exons making up the α_{IIb} and β_3 genes, splicing errors also contribute to the known defects. Identified molecular defects in the gene for GPIIb are listed in Table 42.3 and those in GPIIIa in Table 42.4. Although there is still no X-ray crystallographic structure available for $\alpha_{IIb}\beta_3$, computer modelling by homology with structures available from other integrins starts to give some insight into a general structure for $\alpha_{IIb}\beta_3$ and how it might function[69]. Thus, many of the mutations in α_{IIb} that lead to absence of expression or to defective function while partial expression of the complex is maintained are in a seven blade β-propeller structure[70] which is predicted to be the ligand binding site of this subunit (Fig. 42.2). Some of these involve calcium binding domains which are in the fourth to seventh blades of the propeller. The mutations often change the charge of a residue. These mutations include G273D (numbering based on the mature protein), before the first calcium binding domain[71], E324K[72] and R327H[73] between the second and third calcium-binding domains, G418D before the fourth calcium-binding domain[74] and a V425D426 deletion at the beginning of the fourth calcium-binding domain[75]. A cluster of mutations (P145A, P145L[76], T176I[77] and L183P[78]) lie in the third blade of the propeller implicated to participate in ligand-binding and cause receptor dysfunction. A further engineered mutation, D224V, in a connecting strand within this structural domain also led to GT[79].

While α_{IIb} subunit expression is restricted to $\alpha_{IIb}\beta_3$ and mutations and deletions of this subunit only affect this integrin, mutations and deletions of the β_3 subunit affect both $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$. A large number of mutations and deletions lead to frame shifts and premature termination and hence to failure to express the integrin complex. One might expect that patients with GT caused by β_3 defects could show additional problems. In a mouse model, where the β_3 gene was ablated, an osteosclerotic phenotype was observed in ageing mice with marked bone thickening, presumably due to loss of $\alpha_v\beta_3$ on osteoclasts[80]. No additional effects on platelet function or on angiogenesis were detected. Within the β_3 subunit two major structural units have been identified. One of these is the cation-binding domain, classed as a MIDAS structure or I-domain-like structure[81] and the other is the region rich in disulfide bonds recently recognized as having a protein disulfide isomerase function[82]. Within the MIDAS domain eight point mutations leading to amino acid replacements causing GT have been identified (Fig. 42.3). Two of these, D119Y[83] and D119N[84] are in the DXSXS motif, which is highly conserved. Three mutations, R214W[85], R214Q[86] and

Cambridge Books Online © Cambridge University Press, 2009

Table 42.3. Mutations in GPIIb leading to Glanzmann's thrombasthenia

Exon	Mutation	Phenotype	RNA splicing	AA substitution	Genotype	Patient	Ref
1	IVS1-9del4.5kb	Del: out of frame	Alternative	Premature termination	Homozygote	KW	121
2	288delC	Del: out of frame		Premature termination	Homozygote	Spain-2	122
4	IVS3(-3)-418del	Del: In frame	Alternative	A(106)-Q(111)del	Homozygote	Arab	123
4	480C>G	Del: in frame	Alternative	S(129)-S(161)del	Compound heterozygote	CW	124
4	526C>G	Missense		P176A(P145A)	Compound heterozygote	JF	76
4	526C>T	Missense		P176A(P145A)	Homozygote	Mennonite	76
4	527C>T	Missense		P176L(P145L)	Compound heterozygote	Chinese-14	76
5	IVS5(+2)C>A	Ins: out of frame	Alternative	Premature termination	Compound heterozygote	Spain-1	125
5	575–580ins	Ins: in frame		R192T193(R161T162	Homozygote	KO	126
5	620C>T	Missense		T207I(T176I)	Homozygote	Frankfurt I	127
6	641T>C	Missense		L214P(L183P)	Homozygote	LW	78
8	818G>A	Missense		G273D(G242D)	Homozygote	FLD	71
11	959T>C	Missense		F320S(F289S	Compound heterozygote	Japanese-1	128
12	1063G>A	Missense		E355K(E324K)	Homozygote/	FL	129
					Compound heterozygote	Japanese-2/Swiss	72
12	1073G>A	Missense		R358H(R327H)	Homozygote	KJ/Mila-1	73
13	1346G>A	Missense		G449D(G418D)	Homozygote	LM	74
13	1366–1371del	Del: in frame		V(425)D(426)del	Compound heterozygote	LeM	75
14	1413C>G	Nonsense		Y471X(Y440X)	Compound heterozygote	DV/SV	130
15	IVS15(+1)G>A	Del: out of frame	Alternative	Premature termination	Homozygote	Gypsy	131
16	IVS15(-1)del	Unknown			Compound heterozygote	Chinese-14	76
17	1750C>T	Nonsense		R584X(R553X)		Chinese-10/CW/Family II	124
						Family L/MO/SK	132,133
18	IVS17(-1)G>A	Del: in frame	Alternative	D(585)-Q(626)del	Compound heterozygote	MS	134
18	1787T>C	Missense		I596T(I565T)	Compound heterozygote	CG/Swiss	72
20	IVS19(-2)A>G	Del: out of frame	Alternative	Premature termination	Homozygote	I-J3	135
21	2113T>C	Missense		C705R(C674R)	Compound heterozygote	Spain-1	125
23	2333A>C	Missense		Q778P(Q747P)	Compound heterozygote	Japanese-3/-4/MS/MT	128
25	2473–2478del/ins	Del/ins: in frame	Alternative	L(786)-(795)del>ins	Homozygote	Iran-Jewish	136
26	IVS25(-3)C>G	Del: in frame	Alternative	V868–V909del	Compound heterozygote	SK	133
26	2609C>A	Nonsense		S901X(S870X)	Homozygote	SS	137
28	2929C>T	Nonsense		R977X(R946X)	Compound heterozygote	JF	76
28	2941C>T	Nonsense	Alternative	Q(950X)>P(917–950)del	Compound heterozygote	NR	138
29	IVS29(+2)T>C	Del: in frame	Alternative	V(951)-K(989)del	Compound heterozygote	MC	124
29	3015insG	Ins: out of frame		Frameshift	Compound heterozygote	DV/SV	130
30	IVS29(+2)T>C	Del: in frame	Alternative	V(951)-K(989)del	Compound heterozygote	CG	124
30	3077G>A	Missense		R1026Q(R995Q)	Compound heterozygote	AP	139
30	3094TGins	Ins: out of frame		Frameshift	Compound heterozygote	MC	124

Cambridge Books Online © Cambridge University Press, 2009

Table 42.4. Mutations in GPIIIa leading to Glanzmann's thrombasthenia

Exon	Mutation	Phenotype	RNA splicing	AA substitution	Genotype	Patient	Ref
1	IVS1-5Aluinv15kb + IVS1del11kb	Inv/del		No Transcript	Compound heterozygote	GT3	[140,141]
2	IVS2(+1)G>T	Del: out of frame	Alternative	Premature termination	Homozygote	Amsterdam I	[142]
3	262C>T	Missense		R88X(R62X)	Homozygote	Family I	[143]
4	428T>G	Missense		L143W(L117W)	Homozygote	MK	[88]
4	433G>A	Missense		D145N(D119N)	Homozygote	NR-1	[84]
4	433G>T	Missense		D145Y(D119Y)	Homozygote	Cam	[83]
4	563C>T	Missense		S188L(S162L)	Homozygote	BL	[89]
5	IVS5(+1)G>A	Del/ins: out of frame	Alternative	Premature termination	Compound heterozygote	GT3	[140]
5	718C>T	Missense		R240W(R214W)	Homozygote	CM/Strasbourg I	[85]
5	719G>A	Missense		R240Q(R214Q)	Homozygote	ET	[144,145]
5	725G>A	Missense		R242Q(R216Q)	Homozygote	SH	[87]
6	847delGC	Del: out of frame		Premature termination	Compound heterozygote	LD	[90]
6	863T>C	Missense		L288P(L262P	Compound heterozygote	LD	[90]
6	917A>C	Missense		H306P(H280P)	Compound heterozygote	HJ/NT/TK	[146]
8	1053–1058del	Del/ins: in frame		351–353del M351ins	Homozygote	HS	[147]
9	IVS9Alu-2163 or 2166del11.2kb	Del: out of frame		Premature termination	Homozygote	IJ2	[148]
9	IVS9ins3–4kb	Ins: out of frame		No transcript	Homozygote	CB	[149]
9	1199G>A	Missense		C400Y(C374Y)	Homozygote	Chinese 20	[91]
9	1260G-A + 1143C>A	Del/ins: in frame	Alternative	K(350)-S(396)del/V(350)S(351)ins	Homozygote	RS	[150]
11	1702T>C	Missense		C568R(C542R)	Homozygote	I.M.(Swiss II)	[92]
11	1757G>T	Missense		C586F(C560F)	Compound heterozygote	HJ	[146]
11	1791delT	Del: out of frame		No transcript	Compound heterozygote	RM	[93]
11	1813G>A	Missense		G605S(G579S)	Compound heterozygote	TK	[146]
12	1924G>T	Nonsense		E642X(E616X)	Homozygote	Spain	[151]
13	2031–2041del	Del: out of frame		Premature termination	Homozygote	IJ1	[123]
14	2248C>T	Missense		R750X(R724X)	Compound heterozygote	RM	[93]
15	2332T>C	Missense		S778P(S752P)	Compound heterozygote	P/Paris I	[94]

Cambridge Books Online © Cambridge University Press, 2009

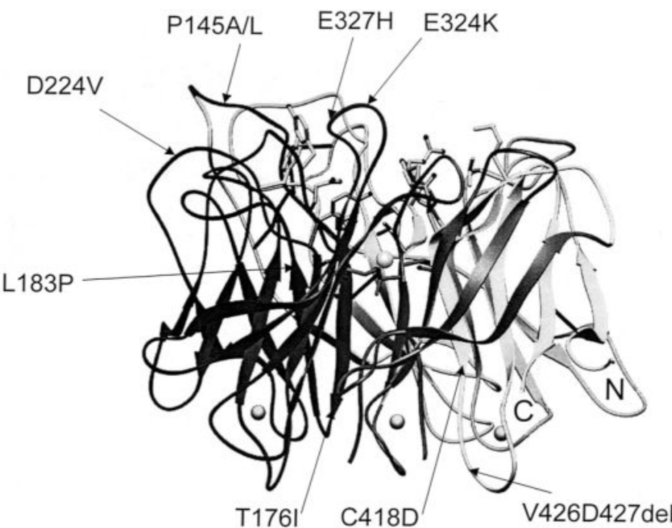


Fig. 42.2. Model of the outer domain of the α_{IIb} subunit as a β-propeller, indicating the position of amino acid substitutions/deletions involved in Glanzmann's thrombasthenia. This domain is predicted to be the ligand binding region of the IIb subunit. There are seven blades, consisting of antiparallel β-folds connected by loops, which include four calcium binding domains towards the bottom of the structure.

R216Q[87] are near cation coordinating sites, and three mutations, D117W[88], S162L[89] and L262P[90] are in the MIDAS structural unit. It is interesting that while the mutations D119Y and D119N affect function but have little effect on expression that at D117W prevents surface expression. The mutations near the cation coordinating sites, R214W, R214Q and R216Q, increase susceptibility of $\alpha_{IIb}\beta_3$ complexes containing these mutated β_3 subunits to dissociation by calcium chelating agents. The mutations at S162L and L262P cause both reduced surface expression (~30%) as well as increased sensitivity of $\alpha_{IIb}\beta_3$ to dissociation by calcium. The S162L mutation results in a complex which does not bind fibrinogen but does support clot retraction, implying that the complex recognizes fibrin. The recently described L262P mutation caused reduced expression and easily dissociated complexes. Again, cells expressing the mutant complex were able to sustain clot retraction while failing to bind to immobilized fibrinogen. Mutations in cysteine residues within the β_3 subunit are also critical for folding and complex formation. These include C374Y[91] and C457R[15] which lead to decreased amounts of expression and C542R[92] where no surface expression was detected.

A great deal of interest has been raised by mutations in the cytoplasmic domain of β_3 which affect inside-out signalling and activation of the receptor for ligand binding. One of these in the codon for R724 to a nonsense triplet causes premature termination and deletion of the C-terminal 39 amino acids[93]. Another is an S752P mutation[94]. Although platelets from both patients express $\alpha_{IIb}\beta_3$ they do not aggregate in response to platelet agonists. Nevertheless, they adhere to immobilized fibrinogen but do not spread on this surface. These mutations do not affect fibrinogen uptake suggesting that outside-in signalling via $\alpha_{IIb}\beta_3$ is not necessary for this function.

GT is the commonest of the platelet receptor related disorders and one of the best documented. An Internet database has been established with data on patients and the possibility of contributing new data (*http://med.mssm.edu/glanzmanndb*). It is to be hoped that, in the future, this will be extended to cover other platelet-related defects.

$\alpha_2\beta_1$ deficiency

In the 1980s two patients were described with mild bleeding problems related to a deficient expression of $\alpha_2\beta_1$[95,96]. These patients had a defective response to collagen but not to other agonists and both surface labelling studies as well as two-dimensional gel electrophoresis studies demonstrated low levels or absent $\alpha_2\beta_1$. In one case further studies showed decreased adhesion to vascular subendothelium as well as poor activation and spreading. Both patients were women and one passed through the menopause soon after these studies which was linked to a recovery of responses to collagen. The other was younger at that time, but has also recently passed the menopause and also showed a recovery of platelet function. It is also seems likely that both these patients started to show symptoms only after their menarche. Other studies found a wide distribution of levels of $\alpha_2\beta_1$ expression among normal donors linked to silent polymorphisms in the gene for α_2[97] so it remains unclear to what extent the two patients were abnormal. It is possible that they represent extreme levels within the 'normal' range perhaps coupled to a low 'normal' value of a coagulation factor, together leading to an enhanced bleeding tendency. The recovery after the menopause suggests a role of hormones in expression levels which is not implausible given the hormone dependency of some transduction factors. These studies suggest that loss of, or inhibition of, $\alpha_2\beta_1$ might not cause major bleeding problems and that inhibition of this receptor might be a possible antithrombotic strategy. However, $\alpha_2\beta_1$ is expressed on vascular cells such as endothelial and smooth muscle cells as well as platelets and it is not known

Cambridge Books Online © Cambridge University Press, 2009

Fig. 42.3. Model of the outer domain of the β_3 subunit as a MIDAS/I-domain, indicating the position of amino acid substitutions involved in Glanzmann's thrombasthenia. The structure is based upon the folding pattern found in several such domains including the A domain of von Willebrand factor and the I-domain of the α_2 integrin subunit.

if these were affected in the patients, presenting an additional complication.

In 1995 a patient suffering from a myeloproliferative disorder was shown to have a specific deficiency in platelet $\alpha_2\beta_1$ and lacked aggregation and adhesion responses to collagen[98]. The patient had a prolonged bleeding time and marked thrombocytosis.

GPVI deficiency

The detection of patients lacking or deficient in GPVI was an important step in identifying this glycoprotein as a critical collagen receptor. Although GPVI was recognized earlier on platelets its function was obscure. The first patient to be identified had platelets with a specific collagen-response defect and her plasma contained antibodies that recognized GPVI in normal platelets[99]. It is still not clear why this patient developed an antibody to an absent receptor but results from mouse model studies start to cast some light on possible explanations. The antibodies from this patient were extremely useful in further characterization of GPVI but also helped to identify other Japanese patients with a defect in this receptor. At least three other patients were found, of whom two also completely lacked surface expression of GPVI while the third had 10% of normal amounts[100,101]. Although both the gene sequence[102] as well as the genomic structure of GPVI[103] are now established, the molecular reason for the absence of GPVI in these patients has not yet been established. Since expression of GPVI and its signalling subunit Fcγ are closely linked[104], it cannot be excluded that the defect may lie in

Cambridge Books Online © Cambridge University Press, 2009

the gene for Fcγ. Some recent studies in mice with a rat monoclonal antibody to GPVI may also provide an explanation for the patient with the autoantibodies by showing that there is down-regulation of GPVI expression on normal mouse platelets in the presence of this antibody[105]. Patients with GPVI deficient platelets have so far only been detected in Japan. Although this may mean that the deficiency is restricted to, or more common, in the Japanese, it is also possible that the mild bleeding tendency, that has permitted its detection, is not present in other ethnic groups with more thrombogenic diets and lifestyles. A wider screening of other population groups may detect more GPVI deficient individuals.

CD36 deficiency

CD36 is the general name for a molecule that was also named GPIIIb or GPIV in platelets. Many early studies on this glycoprotein were also carried out on platelets. There was considerable interest when CD36 was found to be absent in a small population (4–7%) of healthy donors. At first this deficiency was thought to be confined to the Japanese but then other deficient individuals were found in different East Asian populations. More recent studies showed that CD36 is also absent in a minority (7–10%) of the sub-Saharan populations of Africa and in a very small part (~0.3%) of populations in other parts of the world. Is CD36 an adhesive receptor? This has been a controversial area and is even now not completely resolved. Two major adhesive proteins, collagen[106] and thrombospondin[107] have been suggested as ligands for CD36, but it is clear that other proteins also bind to this receptor, including *Plasmodium*-infected erythrocytes and that its main function may be as a scavenger for oxidised lipoproteins[108] or a transporter of long-chain fatty acids[109]. Other authors found little evidence for a role of CD36 as a collagen receptor[110,111]. The molecular basis of CD36 deficiency has been identified as a polymorphism in codon 90 which, if expressed, would lead to a Ser$\rightarrow$Pro shift[112].

Other putative candidates for adhesive receptor defects

Platelets have a number of other receptors which are thought to have either a direct role in adhesion such as $\alpha_5\beta_1$ and $\alpha_6\beta_1$, or a supporting role, such as CD9 or CD47 but so far no bleeding disorders have been linked to a defect in any of these. This may be because they have relatively minor roles in platelet function or, alternatively, because they have major functions on platelets or other cells during embryonal development. Targeted ablation of the genes for these receptors in a tissue-specific way will provide more information about the possible consequences of natural defects. In the long-term proteomic approaches to the analysis of platelets from patients with mild bleeding disorders, related to platelet defects, may be expected to identify a variety of new receptor-linked disorders, whether in the receptors themselves or in downstream signalling pathways.

Acknowledgement

Work performed at the Theodor Kocher Institute was supported by a grant from the Swiss National Science Foundation #31–52396.97

References

1 Lopez JA, Chung DW, Fujikawa K, Hagen FS, Papayannopoulou T, Roth GJ. Cloning of the α chain of human platelet glycoprotein Ib: a transmembrane protein with homology to leucine-rich α 2– glycoprotein. *Proc Natl Acad Sci USA* 1987; 84: 5615–5619.
2 Lopez JA, Chung DW, Fujikawa K, Hagen FS, Davie EW, Roth GJ. The alpha and beta chains of human platelet glycoprotein Ib are both transmembrane proteins containing a leucine-rich amino acid sequence. *Proc Natl Acad Sci USA* 1988; 85: 2135–2139.
3 Hickey MJ, Williams SA, Roth GJ. Human platelet glycoprotein IX: an adhesive prototype of leucine-rich glycoproteins with flank-center-flank structures. *Proc Natl Acad Sci USA* 1989; 86: 6773–6777.
4 Bernard J, Soulier JP. Sur une nouvelle variété de dystrophie thrombocytaire-hémorragipare congénitale. *Semin Hôp Paris* 1948; 24: 3217–3223.
5 Nurden AT, Caen JP. Specific roles for platelet surface glycoproteins in platelet function. *Nature* 1975; 255: 720–722.
6 Jenkins CS, Phillips DR, Clemetson KJ, Meyer D, Larrieu MJ, Luscher EF. Platelet membrane glycoproteins implicated in ristocetin-induced aggregation. Studies of the proteins on platelets from patients with Bernard–Soulier syndrome and von Willebrand's disease. *J Clin Invest* 1976; 57: 112–124.
7 Phillips DR, Agin PP. Platelet plasma membrane glycoproteins. Evidence for the presence of nonequivalent disulfide bonds using nonreduced-reduced two-dimensional gel electrophoresis. *J Biol Chem* 1977; 252: 2121–2126.
8 Clemetson KJ, McGregor JL, James E, Dechavanne M, Luscher EF. Characterization of the platelet membrane glycoprotein

Cambridge Books Online © Cambridge University Press, 2009

abnormalities in Bernard–Soulier syndrome and comparison with normal by surface-labeling techniques and high-resolution two-dimensional gel electrophoresis. *J Clin Invest* 1982; 70: 304–311.

9 Berndt MC, Gregory C, Chong BH, Zola H, Castaldi PA. Additional glycoprotein defects in Bernard–Soulier's syndrome: confirmation of genetic basis by parental analysis. *Blood* 1983; 62: 800–807.

10 Lanza F, Morales M, de La Salle C et al. Cloning and characterization of the gene encoding the human platelet glycoprotein V. A member of the leucine-rich glycoprotein family cleaved during thrombin-induced platelet activation. *J Biol Chem* 1993; 268: 20801–20807.

11 Kahn ML, Diacovo TG, Bainton DF, Lanza F, Trejo J, Coughlin SR. Glycoprotein V-deficient platelets have undiminished thrombin responsiveness and do not exhibit a Bernard–Soulier phenotype. *Blood* 1999; 94: 4112–4121.

12 Ramakrishnan V, Reeves PS, DeGuzman F et al. Increased thrombin responsiveness in platelets from mice lacking glycoprotein V. *Proc Natl Acad Sci USA* 1999; 96: 13336–13341.

13 Ni H, Ramakrishnan V, Papalia JM, Phillips DR, Wagner DD. Increased thrombogenesis and embolus formation in mice lacking glycoprotein V. *Blood* 2000; 96: 812a.

14 Lopez JA, Andrews RK, Afshar-Kharghan V, Berndt MC. Bernard–Soulier syndrome. *Blood* 1998; 91: 4397–4418.

15 Nurden AT. Inherited abnormalities of platelets. *Thromb Haemost* 1999; 82: 468–480.

16 Miller JL, Lyle VA, Cunningham D. Mutation of leucine-57 to phenylalanine in a platelet glycoprotein Ibα leucine tandem repeat occurring in patients with an autosomal dominant variant of Bernard–Soulier disease. *Blood* 1992; 79: 439–446.

17 Clemetson JM, Kyrle PA, Brenner B, Clemetson KJ. Variant Bernard–Soulier syndrome associated with a homozygous mutation in the leucine-rich domain of glycoprotein IX. *Blood* 1994; 84: 1124–1131.

18 Jamieson GA, Okumura T. Reduced thrombin binding and aggregation in Bernard–Soulier platelets. *J Clin Invest* 1978; 61: 861–884.

19 Bevers EM, Comfurius P, Nieuwenhuis HK et al. Platelet prothrombin converting activity in hereditary disorders of platelet function. *Br J Haematol* 1986; 63: 335–345.

20 De Candia E, Hall SW, Rutella S, Landolfi R, Andrews RK, De Cristofaro R. Binding of thrombin to the Glycoprotein Ib accelerates the hydrolysis of PAR-1 on intact platelets. *J Biol Chem* 2001; 276: 4692–4698.

21 Li CQ, Vindigni A, Sadler JE, Wardell MR. Platelet glycoprotein Ib-α binds to thrombin anion-binding exosite II inducing allosteric changes in thrombin's activity. *J Biol Chem* 2001; 276: 6161–6168.

22 Dormann D, Clemetson KJ, Kehrel BE. The GPIb thrombin-binding site is essential for thrombin-induced platelet procoagulant activity. *Blood* 2000; 96: 2469–2478.

23 White JG, Burris SM, Hasegawa D, Johnson M. Micropipette aspiration of human blood platelets: a defect in Bernard–Soulier's syndrome. *Blood* 1984; 63: 1249–1252.

24 Simsek S, Admiraal LG, Modderman PW, van der Schoot CE, Von dem Borne AEGK. Identification of a homozygous single base pair deletion in the gene coding for the human platelet glycoprotein Ibα causing Bernard–Soulier syndrome. *Thromb Haemost* 1994; 72: 444–449.

25 Ware J, Russell SR, Vicente V et al. Nonsense mutation in the glycoprotein Ibα coding sequence associated with Bernard–Soulier syndrome. *Proc Natl Acad Sci USA* 1990; 87: 2026–2030.

26 Kunishima S, Miura H, Fukutani H et al. Bernard–Soulier syndrome Kagoshima: Ser 444$\rightarrow$stop mutation of glycoprotein (GP) Ibα resulting in circulating truncated GPIbα and surface expression of GPIbβ and GPIX. *Blood* 1994; 84: 3356–3362.

27 Holmberg L, Karpman D, Nilsson I, Olofsson T. Bernard–Soulier syndrome Karlstad: Trp 498$\rightarrow$Stop mutation resulting in a truncated glycoprotein Ibα that contains part of the transmembranous domain. *Br J Haematol* 1997; 98: 57–63.

28 Noda M, Fujimura K, Takafuta T et al. Heterogeneous expression of glycoprotein Ib, IX and V in platelets from two patients with Bernard–Soulier syndrome caused by different genetic abnormalities. *Thromb Haemost* 1995; 74: 1411–1415.

29 Li C, Pasquale DN, Roth GJ. Bernard–Soulier syndrome with severe bleeding: absent platelet glycoprotein Ibα due to a homozygous one-base deletion. *Thromb Haemost* 1996; 76: 670–674.

30 Kanaji T, Okamura T, Kurolwa M et al. Molecular and genetic analysis of two patients with Bernard–Soulier syndrome – identification of new mutations in glycoprotein Ibα gene. *Thromb Haemost* 1997; 77: 1055–1061.

31 Afshar-Kharghan V, Craig FE, Lopez JA. Bernard–Soulier syndrome in a patient doubly heterozygous for two frameshift mutations in the glycoprotein Ibα gene. *Br J Haematol* 2000; 110: 919–924.

32 Arai M, Yamamoto N, Akamatsu N et al. Substantial expression of glycoproteins IX and V on the platelet surface from a patient with Bernard–Soulier syndrome. *Br J Haematol* 1994; 87: 185–188.

33 Kenny D, Morateck PA, Gill JC, Montgomery RR. The critical interaction of glycoprotein (GP) Ibβ with GPIX – a genetic cause of Bernard–Soulier syndrome. *Blood* 1999; 93: 2968–2975.

34 Moran N, Morateck PA, Deering A et al. Surface expression of glycoprotein Ibα is dependent on glycoprotein Ibβ: evidence from a novel mutation causing Bernard–Soulier syndrome. *Blood* 2000; 96: 532–539.

35 Simsek S, Noris P, Lozano M et al. Cys209 Ser mutation in the platelet membrane glycoprotein Ibα gene is associated with Bernard–Soulier syndrome. *Br J Haematol* 1994; 88: 839–844.

36 Rivera CE, Villagra J, Riordan M, Williams S, Lindstrom KJ, Rick ME. Short Report: Identification of a new mutation in platelet glycoprotein IX (GPIX) in a patient with Bernard–Soulier syndrome. *Br J Haematol* 2001; 112: 105–108.

Cambridge Books Online © Cambridge University Press, 2009

37 Noda M, Fujimura K, Takafuta T et al. A point mutation in glycoprotein IX coding sequence (Cys73 (TGT) to Tyr(TAT)) causes impaired surface expression of GPIb/IX/V complex in two families with Bernard–Soulier syndrome. *Thromb Haemost* 1996; 76: 874–878.

38 Kunishima S, Tomiyama Y, Honda S et al. Cys97→Tyr mutation in the glycoprotein IX gene associated with Bernard–Soulier syndrome. *Br J Haematol* 1999; 107: 539–545.

39 Kunishima S, Lopez JA, Kobayashi S et al. Missense mutations of the glycoprotein (GP) Ibβ gene impairing the GPIb α/β disulfide linkage in a family with giant platelet disorder. *Blood* 1997; 89: 2404–2412.

40 Li C, Martin SE, Roth GJ. The genetic defect in two well-studied cases of Bernard–Soulier syndrome: a point mutation in the fifth leucine-rich repeat of platelet glycoprotein Ibα. *Blood* 1995; 86: 3805–3814.

41 Noris P, Arbustini E, Spedini P, Belletti S, Balduini CL. A new variant of Bernard–Soulier syndrome characterized by dysfunctional glycoprotein (GP) Ib and severely reduced amounts of GPIX and GPV. *Br J Haematol* 1998; 103: 1004–1013.

42 Wright SD, Michaelides K, Johnson DJD, West NC, Tuddenham EGD. Double heterozygosity for mutations in the platelet glycoprotein IX gene in three siblings with Bernard–Soulier syndrome. *Blood* 1993; 81: 2339–2347.

43 Noris P, Simsek S, Stibbe J, von dem Borne AE. A phenylalanine-55 to serine amino-acid substitution in the human glycoprotein IX leucine-rich repeat is associated with Bernard–Soulier syndrome. *Br J Haematol* 1997; 97: 312–320.

44 Strassel C, Lanza F, De La Salle C et al. A novel missense mutation in GPIbβ prevents the normal maturation and surface expression of the GPIb–IX–V complex in a patient with the Bernard–Soulier syndrome. *Blood* 2000; 96: 816a.

45 Koskela S, Javela K, Jouppila J et al. Variant Bernard–Soulier syndrome due to homozygous Asn45Ser mutation in the platelet glycoprotein (GP) IX in seven patients of five unrelated Finnish families. *Eur J Haematol* 1999; 62: 256–264.

46 Ware J, Russell SR, Marchese P et al. Point mutation in a leucine-rich repeat of platelet glycoprotein Ibα resulting in the Bernard–Soulier syndrome. *J Clin Invest* 1993; 92: 1213–1220.

47 Ulsemer P, Lanza F, Baas MJ et al. Role of the leucine-rich domain of platelet GPIbα in correct post-translational processing – the Nancy I Bernard–Soulier mutation expressed on CHO cells. *Thromb Haemost* 2000; 84: 104–111.

48 Savoia A, Balduini CL, Savino M et al. Autosomal dominant macrothrombocytopenia in Italy is most frequently a type of heterozygous Bernard–Soulier syndrome. *Blood* 2001; 97: 1330–1335.

49 Kunishima S, Tomiyama Y, Honda S et al. Homozygous Pro74→Arg mutation in the platelet glycoprotein Ibβ gene associated with Bernard–Soulier syndrome. *Thromb Haemost* 2000; 84: 112–117.

50 Ludlow LB, Schick BP, Budarf ML et al. Identification of a mutation in a GATA binding site of the platelet glycoprotein Ibβ promoter resulting in the Bernard–Soulier syndrome. *J Biol Chem* 1996; 271: 22076–22080.

51 Miller JL, Cunningham D, Lyle VA, Finch CN. Mutation in the gene encoding the α chain of platelet glycoprotein Ib in platelet-type von Willebrand disease. *Proc Natl Acad Sci USA* 1991; 88: 4761–4765.

52 Russell SD, Roth GJ. Pseudo-von Willebrand disease: a mutation in the platelet glycoprotein Ibα gene associated with a hyperactive surface receptor. *Blood* 1993; 81: 1787–1791.

53 Takahashi H, Murata M, Moriki T et al. Substitution of Val for Met at residue 239 of platelet glycoprotein Ibα in Japanese patients with platelet-type von Willebrand disease. *Blood* 1995; 85: 727–733.

54 Dong J, Schade AJ, Romo GM et al. Novel gain-of-function mutations of platelet glycoprotein Ibα by valine mutagenesis in the Cys209–Cys248 disulfide loop. Functional analysis under statis and dynamic conditions. *J Biol Chem* 2000; 275: 27663–27670.

55 Bolin RB, Okumura T, Jamieson GA. New polymorphism of platelet membrane glycoproteins. *Nature* 1977; 269: 69–70.

56 McGregor JL, Brochier J, Wild F et al. Monoclonal antibodies against platelet membrane glycoproteins. Characterization and effect on platelet function. *Eur J Biochem* 1983; 131: 427–436.

57 Meyer M, Schellenberg I. Platelet membrane glycoprotein Ib: genetic polymorphism detected in the intact molecule and in proteolytic fragments. *Thromb Res* 1990; 58: 233–242.

58 Lopez JA, Ludwig EH, McCarthy BJ. Polymorphism of human glycoprotein Ibα results from a variable number of tandem repeats of a 13-amino acid sequence in the mucin-like macroglycopeptide region. Structure/function implications. *J Biol Chem* 1992; 267: 10055–10061.

59 Ishida F, Furihata K, Ishida K et al. The largest isoform of platelet membrane glycoprotein Ibα is commonly distributed in eastern Asian populations. *Thromb Haemost* 1996; 76: 245–247.

60 Muckian C, Hillmann A, Kenny D, Shields DC. A novel variant of the platelet glycoprotein Ibα macroglycopeptide region lacks any copies of the 'perfect' 13 amino acid repeat [letter]. *Thromb Haemost* 2000; 83: 513–514.

61 Murata M, Matsubara Y, Kawano K et al. Coronary artery disease and polymorphisms in a receptor mediating shear stress-dependent platelet activation. *Circulation* 1997; 96: 3281–3286.

62 Gonzalez-Conejero R, Lozano ML, Rivera J et al. Polymorphisms of platelet membrane glycoprotein Ib associated with arterial thrombotic disease. *Blood* 1998; 92: 2771–2776.

63 Carter AM, Catto AJ, Bamford JM, Grant PJ. Platelet GP IIIa PlA and GP Ib variable number tandem repeat polymorphisms and markers of platelet activation in acute stroke. *Arterioscler Thromb Vasc Biol* 1998; 18: 1124–1131.

64 Ito T, Ishida F, Shimodaira S, Kitano K. Polymorphisms of platelet membrane glycoprotein Ibα and plasma von

Cambridge Books Online © Cambridge University Press, 2009

Willebrand factor antigen in coronary artery disease. *Int J Hematol* 1999; 70: 47–51.

65 Glanzmann E. Hereditäre hämorrhahagische Thrombasthenie: Ein Beitrage zur Pathologie der Blutplättchen. *J Kinderkr* 1918; 88: 113–141.

66 Nurden AT, Caen JP. An abnormal platelet glycoprotein pattern in three cases of Glanzmann's thrombasthenia. *Br J Haematol* 1974; 28: 253–260.

67 Phillips DR, Jenkins CS, Luscher EF, Larrieu M. Molecular differences of exposed surface proteins on thrombasthenic platelet plasma membranes. *Nature* 1975; 257: 599–600.

68 French DL, Seligsohn U. Platelet glycoprotein IIb/IIIa receptors and Glanzmann's thrombasthenia. *Arterioscler Thromb Vasc Biol* 2000; 20: 607–610.

69 Oxvig C, Springer TA. Experimental support for a β-propeller domain in integrin α-subunits and a calcium binding site on its lower surface. *Proc Natl Acad Sci USA* 1998; 95: 4870–4875.

70 Springer TA. Folding of the N-terminal, ligand-binding region of integrin α-subunits into a β-propeller domain. *Proc Natl Acad Sci USA* 1997; 94: 65–72.

71 Poncz M, Rifat S, Coller BS et al. Glanzmann thrombasthenia secondary to a Gly273$\rightarrow$Asp mutation adjacent to the first calcium-binding domain of platelet glycoprotein IIb. *J Clin Invest* 1994; 93: 172–179.

72 Ruan J, Peyruchaud O, Alberio L et al. Double heterozygosity of the GPIIb gene in a Swiss patient with Glanzmann's thrombasthenia. *Br J Haematol* 1998; 102: 918–925.

73 Wilcox DA, Paddock CM, Lyman S, Gill JC, Newman PJ. Glanzmann thrombasthenia resulting from a single amino acid substitution between the second and third calcium-binding domains of GPIIb. Role of the GPIIb amino terminus in integrin subunit association. *J Clin Invest* 1995; 95: 1553–1560.

74 Wilcox DA, Wautier JL, Pidard D, Newman PJ. A single amino acid substitution flanking the fourth calcium binding domain of α_{IIb} prevents maturation of the $\alpha_{IIb}\beta_3$ integrin complex. *J Biol Chem* 1994; 269: 4450–4457.

75 Basani RB, Vilaire G, Shattil SJ, Kolodziej MA, Bennett JS, Poncz M. Glanzmann thrombasthenia due to a two amino acid deletion in the fourth calcium-binding domain of α_{IIb}: demonstration of the importance of calcium-binding domains in the conformation of $\alpha_{IIb}\beta_3$. *Blood* 1996; 88: 167–173.

76 Basani RB, French DL, Vilaire G et al. A naturally occurring mutation near the amino terminus of α_{IIb} defines a new region involved in ligand binding to $\alpha_{IIb}\beta_3$. *Blood* 2000; 95: 180–188.

77 Westrup D, Santoso S, Becker-Hagendorff K et al. Transfection of GPIlbIle176/IIIa(Frankfurt I) in mammalian cells. *Thromb Haemost* 1997; 77: 671.

78 Grimaldi CM, Chen F, Wu C, Weiss HJ, Coller BS, French DL. Glycoprotein IIb Leu214Pro mutation produces Glanzmann thrombasthenia with both quantitative and qualitative abnormalities in GPIIb/IIIa. *Blood* 1998; 91: 1562–1571.

79 Tozer EC, Baker EK, Ginsberg MH, Loftus JC. A mutation in the α subunit of the platelet integrin $\alpha_{IIb}\beta_3$ identifies a novel region important for ligand binding. *Blood* 1999; 93: 918–924.

80 Hynes RO, Hodivala-Dilke KM. Insights and questions arising from studies of a mouse model of Glanzmann thrombasthenia. *Thromb Haemost* 1999; 82: 481–485.

81 Tozer EC, Liddington RC, Sutcliffe MJ, Smeeton AH, Loftus JC. Ligand binding to integrin $\alpha_{IIb}\beta_3$ is dependent on a MIDAS-like domain in the β_3 subunit. *J Biol Chem* 1996; 271: 21978–21984.

82 O'Neill S, Robinson A, Deering A, Ryan M, Fitzgerald DJ, Moran N. The platelet integrin $\alpha_{IIb}\beta_3$ has an endogenous thiol isomerase activity. *J Biol Chem* 2000; 275: 36984–36990.

83 Loftus JC, Te OT, Plow EF, Glass A, Frelinger AL, Ginsberg MH. A β_3 integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. *Science* 1990; 249: 915–918.

84 Ward CM, Chao YL, Kato GJ, Casella J, Bray PF, Newman PJ. Substitution of Asn, but not Tyr, for Asp119 of the β_3 integrin subunit preserves fibrin binding and clot retraction. *Blood* 1997; 90: 26a.

85 Lanza F, Stierlé A, Fournier D et al. A new variant of Glanzmann's thrombasthenia (Strasbourg I). Platelets with functionally defective glycoprotein IIb–IIIa complexes and a glycoprotein IIIa ^{214}Arg$\rightarrow$^{214}Trp mutation. *J Clin Invest* 1992; 89: 1995–2004.

86 Bajt ML, Ginsberg MH, Frelinger ALd, Berndt MC, Loftus JC. A spontaneous mutation of integrin $\alpha_{IIb}\beta_3$ (platelet glycoprotein IIb–IIIa) helps define a ligand binding site. *J Biol Chem* 1992; 267: 3789–3794.

87 Newman PJ, Weyerbusch-Bottum S, Visentin GP, Gidwitz S, White GC. Type II Glanzmann thrombasthenia due to a destabilizing amino acid substitution in platelet membrane GPIIIa. *Thromb Haemost* 1993; 69: 1017.

88 Basani RB, Brown DL, Vilaire G, Bennett JS, Poncz M. A Leu117$\rightarrow$Trp mutation within the RGD-peptide cross-linking region of β_3 results in Glanzmann thrombasthenia by preventing $\alpha_{IIb}\beta_3$ export to the platelet surface. *Blood* 1997; 90: 3082–3088.

89 Jackson DE, White MM, Jennings LK, Newman PJ. A Ser162$\rightarrow$Leu mutation within glycoprotein (GP) IIIa (integrin β_3) results in an unstable $\alpha_{IIb}\beta_3$ complex that retains partial function in a novel form of type II Glanzmann thrombasthenia. *Thromb Haemost* 1998; 80: 42–48.

90 Ward CM, Kestin AS, Newman PJ. A Leu262Pro mutation in the integrin β_3 subunit results in an $\alpha_{IIb}\beta_3$ complex that binds fibrin but not fibrinogen. *Blood* 2000; 96: 161–169.

91 Grimaldi CM, Chen F, Scudder LE, Coller BS, French DL. A Cys374Tyr homozygous mutation of platelet glycoprotein IIIa (β_3) in a Chinese patient with Glanzmann's thrombasthenia. *Blood* 1996; 88: 1666–1675.

92 Ruan J, Schmugge M, Clemetson KJ et al. Homozygous Cys542$\rightarrow$Arg substitution in GPIIIa in a Swiss patient with type I Glanzmann's thrombasthenia. *Br J Haematol* 1999; 105: 523–531.

93 Wang R, Shattil SJ, Ambruso DR, Newman PJ. Truncation of

Cambridge Books Online © Cambridge University Press, 2009

the cytoplasmic domain of beta3 in a variant form of Glanzmann thrombasthenia abrogates signaling through the integrin $\alpha_{IIb}\beta_3$ complex. *J Clin Invest* 1997; 100: 2393–2403.

94 Chen Y-P, Djaffar I, Pidard D et al. Ser-752→Pro mutation in the cytoplasmic domain of integrin β_3 subunit and defective activation of platelet integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb–IIIa) in a variant of Glanzmann thrombasthenia. *Proc Natl Acad Sci USA* 1992; 89: 10169–10173.

95 Nieuwenhuis HK, Akkerman JW, Houdijk WP, Sixma JJ. Human blood platelets showing no response to collagen fail to express surface glycoprotein Ia. *Nature* 1985; 318: 470–472.

96 Kehrel B, Balleisen L, Kokott R et al. Deficiency of intact thrombospondin and membrane glycoprotein Ia in platelets with defective collagen-induced aggregation and spontaneous loss of disorder. *Blood* 1988; 71: 1074–1078.

97 Kunicki TJ, Kritzik M, Annis DS, Nugent DJ. Hereditary variation in platelet integrin $\alpha_2\beta_1$ density is associated with two silent polymorphisms in the α_2 gene coding sequence. *Blood* 1997; 89: 1939–1943.

98 Handa M, Watanabe K, Kawai Y et al. Platelet unresponsiveness to collagen: involvement of glycoprotein Ia–IIa ($\alpha_2\beta_1$ integrin) deficiency associated with a myeloproliferative disorder. *Thromb Haemost* 1995; 73: 521–528.

99 Sugiyama T, Okuma M, Ushikubi F, Sensaki S, Kanaji K, Uchino H. A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia. *Blood* 1987; 69: 1712–1720.

100 Moroi M, Jung SM, Okuma M, Shinmyozu K. A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion. *J Clin Invest* 1989; 84: 1440–1445.

101 Arai M, Yamamoto N, Moroi M, Akamatsu N, Fukutake K, Tanoue K. Platelets with 10% of the normal amount of glycoprotein VI have an impaired response to collagen that results in a mild bleeding tendency. *Br J Haematol* 1995; 89: 124–130.

102 Clemetson JM, Polgar J, Magnenat E, Wells TN, Clemetson KJ. The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcαR and the natural killer receptors. *J Biol Chem* 1999; 274: 29019–29024.

103 Ezumi Y, Uchiyama T, Takayama H. Molecular cloning, genomic structure, chromosomal localization, and alternative splice forms of the platelet collagen receptor glycoprotein VI. *Biochem Biophys Res Commun* 2000; 277: 27–36.

104 Tsuji M, Ezumi Y, Arai M, Takayama H. A novel association of Fc receptor γ-chain with glycoprotein VI and their co-expression as a collagen receptor in human platelets. *J Biol Chem* 1997; 272: 23528–23531.

105 Nieswandt B, Schulte V, Bergmeier W et al. Long-term antithrombotic protection by irreversible inactivation of platelet glycoprotein VI in mice. *Blood* 2000; 96: 269a.

106 Diaz-Ricart M, Tandon NN, Carretero M, Ordinas A, Bastida E, Jamieson GA. Platelets lacking functional CD36 (glycoprotein IV) show reduced adhesion to collagen in flowing whole blood. *Blood* 1993; 82: 491–496.

107 Asch AS, Liu I, Briccetti FM et al. Analysis of CD36 binding domains: Ligand specificity controlled by dephosphorylation of an ectodomain. *Science* 1993; 262: 1436–1440.

108 Endemann G, Stanton LW, Madden KS, Bryant CM, White RT, Protter AA. CD36 is a receptor for oxidized low density lipoprotein. *J Biol Chem* 1993; 268: 11811–11816.

109 Coburn CT, Knapp FF, Jr Febbraio M, Beets AL, Silverstein RL, Abumrad NA. Defective uptake and utilization of long chain fatty acids in muscle and adipose tissues of CD36 knockout mice. *J Biol Chem* 2000; 275: 32523–32529.

110 Kehrel B, Kronenberg A, Rauterberg J et al. Platelets deficient in glycoprotein IIIb aggregate normally to collagens type I and III but not to collagen type V. *Blood* 1993; 82: 3364–3370.

111 Daniel JL, Dangelmaier C, Strouse R, Smith JB. Collagen induces normal signal transduction in platelets deficient in CD36 (platelet glycoprotein IV). *Thromb Haemost* 1994; 71: 353–356.

112 Kashiwagi H, Tomiyama Y, Honda S et al. Molecular basis of CD36 deficiency. Evidence that a 478C→T substitution (proline90→serine) in CD36 cDNA accounts for CD36 deficiency. *J Clin Invest* 1995; 95: 1040–1046.

113 Kenny D, Jonsson OG, Morateck PA, Montgomery RR. Naturally occurring mutations in glycoprotein Ibα that result in defective ligand binding and synthesis of a truncated protein. *Blood* 1998; 92: 175–183.

114 Savoia A, Balduini CL, Savino M et al. Autosomal dominant macrothrombocytopenia in Italy is most frequently a type of heterozygous Bernard–Soulier syndrome. *Blood* 2001; 97: 1330–1335.

115 Margaglione MG, Grandone E, Brancaccio V, Amoriello A, Di Minno G. Compound heterozygosity (554–589 del, C515–T transition) in the platelet glycoprotein Ibα gene in a patient with a severe bleeding tendency. *Thromb Haemost* 1999; 81: 486–492.

116 de la Salle C, Baas MJ, Lanza F et al. A three-base deletion removing a leucine residue in a leucine-rich repeat of platelet glycoprotein Ibα associated with a variant of Bernard–Soulier syndrome (Nancy I). *Br J Haematol* 1995; 89: 386–396.

117 Kunishima S, Miura H, Fukutani H et al. Bernard–Soulier syndrome Kagoshima: Ser 444→stop mutation of glycoprotein (GP) Ibα resulting in circulating truncated GPIbα and surface expression of GPIbβ and GPIX. *Blood* 1994; 84: 3356–3362.

118 Afshar-Kharghan V, Lopez JA. Bernard–Soulier syndrome caused by a dinucleotide deletion and reading frameshift in the region encoding the glycoprotein Ibα transmembrane domain. *Blood* 1997; 90: 2634–2643.

119 Kunishima S, Heaton DC, Naoe T et al. De novo mutation of the platelet glycoprotein Ibα gene in a patient with pseudo-von Willebrand disease. *Blood Coagul Fibrinol* 1997; 8: 311–315.

120 Iwanaga M, Kunishima S, Ikeda S, Tomonaga M, Naoe T. Vulnerable mutation Trp126→stop of glycoprotein IX in Japanese Bernard–Soulier syndrome. *Eur J Haematol* 1998; 60: 264–266.

121 Burk CD, Newman PJ, Lyman S, Gill J, Coller BS, Poncz M. A

Cambridge Books Online © Cambridge University Press, 2009

deletion in the gene for glycoprotein IIb associated with Glanzmann's thrombasthenia. *J Clin Invest* 1991; 87: 270–276.
122 Tao J, Arias-Salgado EG, Gonzalez-Manchon C, Diaz-Cremades J, Ayuso MS, Parrilla R. A novel (288delC) mutation in exon 2 of GPIIb associated with type I Glanzmann's thrombasthenia. *Br J Haematol* 111: 96–103.
123 Newman PJ, Seligsohn U, Lyman S, Coller BS. The molecular genetic basis of Glanzmann thrombasthenia in the Iraqi–Jewish and Arab populations in Israel. *Proc Natl Acad Sci USA* 1991; 88: 3160–3164.
124 French DL, Coller BS. Hematologically important mutations: Glanzmann thrombasthenia. *Blood Cells Mol Dis* 1997; 23: 39–51.
125 Gonzalez-Manchon C, Fernandez-Pinel M, Arias-Salgado EG et al. Molecular genetic analysis of a compound heterozygote for the glycoprotein (GP) IIb gene associated with Glanzmann's thrombasthenia: disruption of the 674–687 disulfide bridge in GPIIb prevents surface exposure of GPIIb–IIIa complexes. *Blood* 1999; 93: 866–875.
126 Honda S, Tomiyama Y, Shiraga M et al. A two-amino acid insertion in the Cys146–Cys167 loop of the α_{IIb} subunit is associated with a variant of Glanzmann thrombasthenia. Critical role of Asp163 in ligand binding. *J Clin Invest* 1998; 102: 1183–1192.
127 Kirchmaier CM, Westrup D, Becker-Hagendorff K, Just M, Jablonka B, Seifried E. A new variant of Glanzmann's thrombasthenia (Frankfurt I). *Thromb Haemost* 1995; 73: 1058.
128 Ambo H, Kamata T, Handa M et al. Novel point mutations in the alphaIIb subunit (Phe289→Ser, Glu324→Lys and Gln747→Pro) causing thrombasthenic phenotypes in four Japanese patients. *Br J Haematol* 1998; 102: 829–840.
129 Bourre R, Peyruchaud O, Bray P, Combrie R, Nurden P, Nurden AT. A point mutation in the gene for platelet GPIIb leads to a substitution in a highly conserved amino acid between the second and the third Ca2+ binding domain. *Blood* 1995; 452a.
130 Scott JP, Scott JP, Chao YL, Newman PJ, Ward CM. A frameshift mutation at Gly975 in the transmembrane domain of GPIIb prevents GPIIb–IIIa expression – analysis of two novel mutations in a kindred with type I Glanzmann thrombasthenia. *Thromb Haemost* 1998; 80: 546–550.
131 Schlegel N, Gayet O, Morel-Kopp MC et al. The molecular genetic basis of Glanzmann's thrombasthenia in a gypsy population in France: identification of a new mutation on the alpha IIb gene. *Blood* 1995; 86: 977–982.
132 Tomiyama Y, Kashiwagi H, Kosugi S et al. Abnormal processing of the glycoprotein IIb transcript due to a nonsense mutation in exon 17 associated with Glanzmann's thrombasthenia. *Thromb Haemost* 1995; 73: 756–762.
133 Kato A, Yamamoto K, Miyazaki S, Jung SM, Moroi M, Aoki N. Molecular basis for Glanzmann's thrombasthenia (GT) in a compound heterozygote with glycoprotein IIb gene: a proposal for the classification of GT based on the biosynthetic pathway of glycoprotein IIb–IIIa complex. *Blood* 1992; 79: 3212–3218.
134 Tadokoro S, Tomiyama Y, Honda S et al. A Gln747→Pro substitution in the IIb subunit is responsible for a moderate IIbβ_3 deficiency in Glanzmann thrombasthenia. *Blood* 1998; 92: 2750–2758.
135 Yatuv R, Rosenberg N, Dardik R, Brenner B, Seligsohn U. Glanzmann thrombasthenia in two Iraqi–Jewish siblings is caused by a novel splice junction mutation in the glycoprotein IIb gene. *Blood Coagul Fibrinol* 1998; 9: 285–288.
136 Peretz H, Rosenberg N, Usher S et al. Glanzmann's thrombasthenia associated with deletion-insertion and alternative splicing in the glycoprotein IIb gene. *Blood* 1995; 85: 414–420.
137 Vinciguerra C, Khelif A, Alemany M et al. A nonsense mutation in the GPIIb heavy chain (Ser 870→stop) impairs platelet GPIIb–IIIa expression. *Br J Haematol* 1996; 95: 399–407.
138 Iwamoto S, Nishiumi E, Kajii E, Ikemoto S. An exon 28 mutation resulting in alternative splicing of the glycoprotein IIb transcript and Glanzmann's thrombasthenia. *Blood* 1994; 83: 1017–1023.
139 Peyruchaud O, Nurden AT, Milet S et al. R to Q amino acid substitution in the GFFKR sequence of the cytoplasmic domain of the integrin IIb subunit in a patient with a Glanzmann's thrombasthenia-like syndrome. *Blood* 1998; 92: 4178–4187.
140 Bray PF, Shuman MA. Identification of an abnormal gene for the GPIIIa subunit of the platelet fibrinogen receptor resulting in Glanzmann's thrombasthenia. *Blood* 1990; 75: 881–888.
141 Li L, Bray PF. Homologous recombination among three intragene Alu sequences causes an inversion-deletion resulting in the hereditary bleeding disorder Glanzmann thrombasthenia. *Am J Hum Genet* 1993; 53: 140–149.
142 Simsek S, Heyboer H, de Bruijne-Admiraal LG, Goldschmeding R, Cuijpers HT, von dem Borne AE. Glanzmann's thrombasthenia caused by homozygosity for a splice defect that leads to deletion of the first coding exon of the glycoprotein IIIa mRNA. *Blood* 1993; 81: 2044–2049.
143 Vinciguerra C, Trzeciak MC, Philippe N et al. Molecular study of Glanzmann thrombasthenia in 3 patients issued from 2 different families. *Thromb Haemost* 1995; 74: 822–827.
144 Fournier DJ, Kabral A, Castaldi PA, Berndt MC. A variant of Glanzmann's thrombasthenia characterized by abnormal glycoprotein IIb/IIIa complex formation. *Thromb Haemost* 1989; 62: 977–983.
145 Bajt ML, Ginsberg MH, Frelinger AL, III Berndt MC, Loftus JC. A spontaneous mutation of integrin $\alpha_{IIb}\beta_3$ (platelet glycoprotein IIb–IIIa) helps define a ligand binding site. *J Biol Chem* 1992; 267: 3789–3794.
146 Ambo H, Kamata T, Handa M et al. Three novel integrin β_3 subunit missense mutations (H280P, C560F, and G579S) in thrombasthenia, including one (H280P) prevalent in Japanese patients. *Biochem Biophys Res Commun* 1998; 251: 763–768.
147 Morel-Kopp MC, Kaplan C, Proulle V et al. A three amino acid deletion in glycoprotein IIIa is responsible for type I Glanzmann's thrombasthenia: importance of residues Ile325Pro326Gly327 for β_3 integrin subunit association. *Blood* 1997; 90: 669–677.

Cambridge Books Online © Cambridge University Press, 2009

148 Rosenberg N, Yatuv R, Orion Y et al. Glanzmann thrombasthenia caused by an 11.2-kb deletion in the glycoprotein IIIa (β_3) is a second mutation in Iraqi Jews that stemmed from a distinct founder. *Blood* 1997; 89: 3654–3662.
149 Djaffar I, Caen JP, Rosa JP. A large alteration in the human platelet glycoprotein IIIa (integrin β_3) gene associated with Glanzmann's thrombasthenia. *Hum Mol Genet* 1993; 2: 2183–2185.
150 Jin Y, Dietz HC, Montgomery RA et al. Glanzmann thrombasthenia. Cooperation between sequence variants in cis during splice site selection. *J Clin Invest* 1996; 98: 1745–1754.
151 Ferrer M, Tao J, Iruin G et al. Truncation of glycoprotein (GP) IIIa (616–762) prevents complex formation with GPIIb: novel mutation in exon 11 of GPIIIa associated with thrombasthenia. *Blood* 1998; 92: 4712–4720.

Cambridge Books Online © Cambridge University Press, 2009

Congenital disorders of platelet secretion

Marco Cattaneo

Angelo Bianchi Bonomi Hemophilia and Thrombosis Centre, IRCCS Ospedale Maggiore, University of Milano, Italy.

A brief introduction on platelet secretion (see Fig. 43.1)

The essential role played by platelets in hemostasis, thrombosis and vascular remodelling is, to a large extent, mediated by effector molecules that they secrete at sites of vascular injury. These molecules are contained in the platelet dense (or δ-) and α-granules. Dense granules are small organelles (200–300 nm in diameter) that are dense to electrons in osmium-stained platelets. They mostly contain small molecules, such as nucleotides, serotonin, catecholamines, Ca^{2+}, Mg^{2+} and pyrophosphate. More recent studies have demonstrated the presence of P-selectin, granulophysin, glycoprotein (GP) Ib and GPIIb/IIIa bound to their membrane[1–3]. Alpha granules, at variance with δ-granules, are moderately electron-dense and have a diameter of about 200–500 nm. The most numerous of the platelet storage organelles, they contain membrane-bound proteins (GPIb/IX/V, GPIIb/IIIa, P-selectin, GPIV, osteonectin, PECAM1, GMP33, CD9) and are the storage site for proteins which are synthesized in the megakaryocytes (von Willebrand factor, thrombospondin, fibronectin, coagulation factor V, platelet-derived growth factor and other growth factors), or acquired from plasma either by receptor-mediated endocytosis (e.g., fibrinogen) or by fluid-phase pynocytosis (e.g., albumin and immunoglobulin G)[4,5].

While δ-granules function primarily for the amplification of platelet activation through the secretion of platelet agonists such as serotonin and adenosine diphosphate (ADP), α- granules enhance the adhesive process, promote cell–cell interactions and stimulate vascular repair. The combined action of molecules secreted by δ- and α-granules contribute to the stabilization of platelet aggregates[6,7].

Other platelet organelles that release their contents in the extracellular fluid upon activation are the lysosomes,

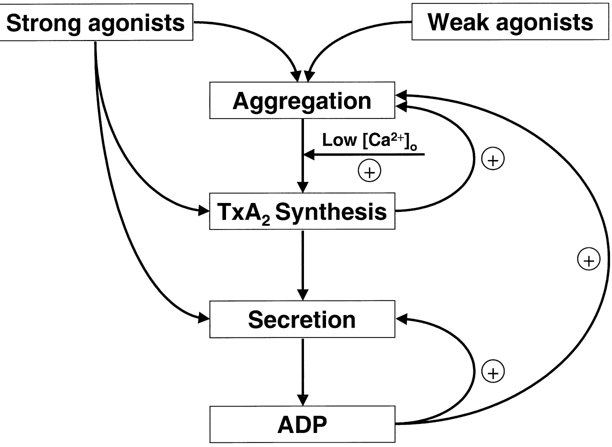


Fig. 43.1. Schematic representation of the amplification pathways of platelet aggregation and secretion. Being a weak agonist, ADP does not induce platelet secretion directly when it interacts with resting platelets. However, once platelet secretion has been triggered by a strong agonist, released ADP potentiates platelet secretion independently of the formation of large platelet aggregates and thromboxane A_2 production[16]. This function of released ADP is mediated by its interaction with the platelet $P2Y_{12}$ receptor[17] (see also Fig. 43.2). ADP, adenosine diphosphate; TxA_2, thromboxane A_2; $[Ca^{2+}]_o$, concentration of external ionized calcium; (+) = potentiation.

which contain degradative enzymes, including acid phosphatases, aryl sulfatase, β-glucuronidase, cathepsin, heparitinase, β-hexosaminidase and β-galactosidase. Their membrane contains several glycoproteins, including CD63, LAMP-1 and LAMP-2. Recent data suggest that lysosome release requires the same membrane fusion machinery that is necessary for δ- and α-granule release[8]. Although lysosome secretion can be easily induced *in vitro* and can occur *in vivo* upon stimulation[9], its physiological relevance is controversial[10]. They may have a role in the

amplification of platelet activation[11] and in vascular remodelling.

Very briefly, the molecular mechanisms of platelet secretion involve the interaction of agonists with their receptors, which are coupled to second messengers, including Ca^{2+}, diacylglycerol and protein kinase C that signal to the SNARE (soluble NSF attachment protein receptor) machinery through yet unknown linkages[12,13] (Chapter 24 in this book). The platelet granules fuse with the membranes of the open canalicular system (OCS), into which they release their contents.

Platelet agonists are usually subdivided in two categories: weak and strong agonists. Weak agonists, such as ADP and epinephrine, induce platelet aggregation, but do not cause platelet secretion directly. However, in a minority of individuals[14], the close platelet-to-platelet contact brought about by platelet aggregation triggers the formation of trace amounts of thromboxane A_2 (TxA_2) which stimulates platelet secretion and, together with substances released from platelet granules, reinforces aggregation. This effect is greatly enhanced and can be observed in most individuals, when the concentration of ionized calcium in the extracellular medium is artefactually decreased to the micromolar level, such as in citrated PRP[14,15]. It must be noted, however, that recent studies have shown that, once platelet secretion has been triggered by a strong agonist, ADP or epinephrine potentiates platelet secretion directly, independently of platelet aggregation and TxA_2 formation[16,17]. In contrast to weak agonists, strong agonists, such as thrombin and collagen, trigger platelet secretion directly, i.e. independently of platelet aggregation and TxA_2 production. With medium-low concentrations of strong agonists, however, some aggregation- and TxA_2-dependent amplification of platelet secretion can be observed, especially at low concentrations of external Ca^{2+}[18,19].

Defects of platelet secretion

Definition

Since platelet secretion and aggregation are intimately related and both depend on platelet activation, a clear distinction between disorders of platelet aggregation, platelet activation and platelet secretion is in many instances problematic. For example, when citrated PRP from patients with Glanzmann's thrombasthenia are stimulated in the aggregometer with ADP, they do not undergo aggregation and, as a consequence, do not undergo the aggregation-dependet secretion. However, Glanzmann's thrombasthenia is generally, and correctly, considered a typical defect of platelet aggregation. This chapter will review those congenital disorders of platelet function in which a secretion defect appears to be the primary or the more relevant abnormality. As a consequence of the secretion defect and the lack of the potentiating effect of released δ-granule constituents, these disorders usually have as a common characteristic an abnormal pattern of aggregation responses to many agonists: (i) absent secondary waves of aggregation, after normal primary waves stimulated by the weak agonists ADP, epinephrine and platelet activating factor (PAF)[20], (ii) impaired aggregation induced by low concentrations of collagen or thrombin. It must be noted, however, that these abnormalities of platelet aggregation, albeit typical, are not to be considered the diagnostic hallmark of defects of platelet secretion, because platelet aggregation is not a sensitive diagnostic test for these abnormalities of platelet function (see later).

Classification

In an attempt to make some order among the very heterogeneous disorders of platelet secretion, they are here subdivided in three groups (Table 43.1): (i) defects of the platelet granules, which could be considered 'pure' defects of platelet secretion; (ii) defects of the two main amplification pathways of platelet activation, the arachidonate-TxA_2 pathway and the ADP pathway; (iii) miscellaneous abnormalities of platelet secretion ('primary secretion defects'): this group encompasses heterogeneous disorders of platelet secretion that cannot be explained by abnormalities of platelet organelles or of the two amplification pathways. Parts of this chapter will inevitably overlap with parts of the following chapter on defects of platelet signal transduction.

Defects of the platelet granules

Defects of the platelet granules comprise a heterogeneous group of disorders, including deficiencies of the δ- and/or α-granules, or their consituents (δ-, α,δ- and α-Storage Pool Deficiency, Table 43.2) and other, less common defects of the α-granules. It must be noted that pure defects of the α-granules do not cause the abnormalities of platelet aggregation that are typical of defects of platelet secretion.

Cambridge Books Online © Cambridge University Press, 2009

Table 43.1. Classification of congenital defects of platelet secretion

Abnormalities of the platelet granules ('Pure' platelet secretion disorders)
δ-storage pool deficiency (δ-SPD)[a]
δ-SPD (not associated to other congenital disorders)
Hermanski–Pudlak syndrome
Chediak–Higashi syndrome
α,δ-storage pool deficiency (α,δ-SPD)
α-storage pool deficiency, or Gray Platelet syndrome (GPS)
Quebec platelet syndrome
Jacobsen or Paris-Trousseau syndrome
Platelet secretion disorders due to abnormalities of the amplification pathways
Abnormalities of the arachidonate/TxA_2 pathway
impaired liberation of arachidonate from membrane phospholipids
defects of cyclooxygenase (aspirin-like defects)
defects of thromboxane synthetase
defects of the platelet TxA_2 receptor
Abnormalities of the ADP pathway
defects of the platelet $P2Y_{12}$ receptor[b]
Miscellaneous abnormalities of platelet secretion ('Primary secretion defects')
Miscellaneous disorders of platelet secretion not due to defects of the platelet granules or of the amplification pathways

Notes:
[a] δ-SPD has also been described in some cases of thrombocytopenia with absent radii (TAR) syndrome and Wiskott–Aldrich syndrome (WAS) (see Chapter 34).
[b] Patients who are homozygous for $P2Y_{12}$ defect, in addition to a secretion defect, have a severe impairment of platelet aggregation induced by ADP.

Defects of the δ-granules (δ-storage pool deficiency)

δ-storage pool deficiency

The term δ-storage pool deficiency, or δ-storage pool disease (δ-SPD) defines a congenital abnormality of platelets characterized by deficiency of dense granules in megakaryocytes and platelets. It is characterized by a bleeding diathesis of variable degree, mildly-to-moderately prolonged skin bleeding time, abnormal platelet secretion induced by several platelet agonists, impaired platelet aggregation and decreased platelet content of dense granules. It may present as an isolated platelet function defect or it may associate with a variety of congenital disorders.

First described in 1969 in a family with impaired platelet aggregation and reduced release of ADP[21], it was later shown to be caused by a deficiency in the nonmetabolic pool of adeninenucleotides contained in the platelet δ-granules[22,23]. Several other patients have been described since then. Between 10% and 18% of patients with congenital abnormalities of platelet function have SPD[24,25]. The inheritance is autosomal recessive in some families and autosomal dominant in others.

Clinical features

Patients with δ-SPD have mild to moderate bleeding diathesis, characterized mainly by mucocutaneous bleedings, such as epistaxis, menorrhagia and easy bruising. Patients with the most severe forms may also experience postsurgical hemorrhagic complications, especially after tooth extraction and tonsillectomy. Only one case with intracranial bleeding has been reported[26]. Hemarthrosis has never been reported in these patients. The bleeding time is usually prolonged, and the extent of its prolongation is inversely related to the amount of ADP or serotonin contained in the granules[27,28].

Studies of a family which includes several members with autosomal dominant δ-SPD showed an association between δ-SPD and the development of acute myelogenous leukemia: on this ground, the hypothesis was raised that a gene coding for a protein important for the formation of dense granules is located adjacent to a gene which, when abnormal, may predispose to the development of leukemia[29,30]. An association of δ-SPD with primary pulmonary hypertension was described in a patient, suggesting a role for the high plasma serotonin levels found in δ-SPD in the pathogenesis of this disorder[31]. The association between pulmonary hypertension and δ-SPD was also described in fawn-hooded rats[32].

Laboratory findings

Studies of δ-granules with uranaffin reaction (staining by uranyl ions of both the δ-granule membrane and core)[33], with the fluorescence probe mepacrine (which concentrates in the δ-granules)[34], or with electron microscopy[35], revealed that platelets from patients with isolated δ-SPD have slightly reduced number of uranaffin-positive and mepacrine-positive granules, but a shift in uranaffin-positive distribution towards those lacking a dense core ('empty granules'), suggesting a more qualitative than quantitative type of δ-granule defect[36]. In accordance with these findings, platelets from patients with isolated

Cambridge Books Online © Cambridge University Press, 2009

Table 43.2. Characteristics of the different forms of congenital storage pool deficiency

			δ-granules		α-granules	
	Clinical features	Inheritance	Content	Membrane	Content	Membrane
δ-storage pool deficiency	Bleeding diathesis	Aut/recessive Aut/dominant	Decreased	Normal	Normal	Normal
Hermansky–Pudlak syndrome	Bleeding diathesis Oculocutaneous albinism Lypofuscin accumulation [complications: pulmonary fibrosis and granulomatous colitis]	Aut/recessive	Decreased	Decreased	Normal	Normal
Chediak–Higashi syndrome	Bleeding diathesis Oculocutaneous albinism Recurrent infections (neutropenia) [accelerated phase: lymphoid infiltration of multiple organs]	Aut/recessive	Decreased	Decreased	Normal	Normal
α,δ-storage pool deficiency	Bleeding diathesis	Aut/recessive	Decreased	Normal (?)	Decreased	Normal (?)
Grey platelet syndrome (α-storage pool deficiency)	Bleeding diathesis Thrombocytopenia Myelofibrosis (splenomegaly)	Aut/recessive Aut/dominant	Normal	Normal	Decreased	Normal

platelet δ-SPD had normal amounts of the δ-granule membrane protein granulophysin[37,38].

δ-SPD platelets have decreased levels of δ-granule constituents: ATP and ADP[22,23], serotonin, calcium and pyrophosphate[39,40]. ADP and ATP are contained in platelets in a metabolic pool, which represents about 1/3 of the total content, and the δ-granules, which represent the storage pool. ADP is present in greater amounts in the storage pool, while the concentration of ATP is higher in the metabolic pool. The ratio of total ATP: ADP in normal platelets is $<2.5:1$, while that of the metabolic pool is about 10: 1. Due to the deficiency of δ-granules, the ratio of total ATP: ADP in δ-SPD platelets typically rises to values >2.5–3: 1[41].

Platelets are the main storage site for serotonin of the human body. Normal platelets avidly uptake serotonin from the bloodstream and store it in the δ-granules, where it is protected from the action of mitochondrial monoamino oxidases. When radioactive serotonin is incubated with normal platelets in vitro, more than 90% of it is rapidly incorporated. In contrast, when it is incubated with δ-SPD platelets, the initial rate of uptake (through the platelet plasma membrane) is normal, but the saturation level is decreased, due to the catabolism of serotonin, resulting in the loss of the radioactive label from the platelets[42].

In citrated platelet-rich plasma, primary aggregation induced by ADP or epinephrine and the agglutination response to ristocetin are normal in these patients, but the second wave of aggregation and the aggregation in response to collagen are generally absent or greatly reduced[23,43]. The production of arachidonate metabolites can be defective after stimulation with epinephrine or collagen, but normal with arachidonate[44]; however, the aggregation induced by sodium arachidonate or prostaglandin endoperoxides may be normal or decreased[43–47], depending on the severity of ADP deficiency in platelet granules[44]. Normal responses to ADP or epinephrine have been observed in some patients[48], indicating that there is a large variability in platelet aggregation in patients with δ-SPD, which has been well documented in a large study of 106 patients with δ-SPD (congenital in ref. 51 and acquired in ref. 55), which showed that about 25% of the patients had normal aggregation responses to ADP, epinephrine and collagen, while only 33% had aggregation tracings typical for a platelet secretion defect[24]. In agreement with these findings, it was later shown that, among 46 patients with prolonged bleeding times, normal vWF levels and normal platelet aggregation 17 (35%) had δ-SPD[49]. Lumiaggregometry, which measures platelet aggregation and secretion simultaneously, may prove a more accurate technique than platelet aggregometry for diagnosing patients with δ-SPD and, more generally, with platelet secretion defects.

Cambridge Books Online © Cambridge University Press, 2009

High concentrations of thrombin induced normal extent of aggregation of δ-SPD platelets, but the aggregates deaggregated more easily than normal[7]. This defect was corrected by the addition of exogenous ADP immediately after thrombin stimulation, suggesting that released ADP plays a role in the stabilization of platelet aggregates[7]. Other platelet function abnormalities described in δ-SPD patients include abnormal secretion of acid hydrolases[50], which was corrected by exogenous ADP[51], and defective aggregation at high shear[28,52].

The in vitro interaction of δ-SPD platelets with the subendothelium was impaired in an experiment of perfusion of citrated blood through a chamber containing everted segments of rabbit aorta[53]. Subsequent experiments performed at different flow conditions (shear rates varying from 650 to 3300 s^{-1}) with non-anticoagulated blood showed that thrombus formation was decreased in δ-SPD patients in proportion to the magnitude of the granule defect[54].

Weiss and Lages showed that the prothrombinase activity induced by collagen, thrombin, or collagen plus thrombin was impaired in δ-SPD platelets and was corrected by the addition of ADP[55]. However, a previous study failed to demonstrate an abnormal procoagulant activity of δ-SPD platelets under slightly different experimental conditions[56].

Diagnosis

The diagnosis of δ-SPD is essentially based on the finding of defective platelet secretion induced by several agonists, decreased platelet content of total ADP and ATP, increase in the ATP/ADP ratio >2.5–3.0, and normal serum concentration of the stable TxA_2 metabolite, TxB_2. Methods involving the identification of mepacrine-loaded platelets by flow cytometry[57,58] or studying platelet aggregation at high shear[28] may also prove to be useful for the diagnosis of this disorder.

The Hermansky–Pudlak syndrome

Clinical features

Hermansky–Pudlak syndrome (HPS) is a rare autosomal recessive disease of subcellular organelles of many tissues, involving abnormalities of melanosomes, platelet δ-granules and lysosomes. It is characterized by tyrosinase-positive oculocutaneous albinism, a bleeding diathesis due to δ-SPD and ceroid-lypofuscin lysosomal storage disease[59]. The oculocutaneous albinism manifests as congenital nystagmus, iris transillumination, decreased visual activity and various degrees of skin and hair hypopigmentation. Ceroid lypufuscin is a lipid–protein complex accumulating in lysosomal organelles, which is believed to be responsible for the development of progressive pulmonary fibrosis[60,61] and granulomatous colitis[62] in affected patients. The pulmonary fibrosis is particularly frequent in patients homozygous for the 16-bp duplication in HPS gene (see below)[63] and usually leads to death within the fourth or fifth decade. In albino patients, the absence of visible δ-granules in the platelet cytoplasm under the electron microscope and/or the deficiency of platelet adenine nucleotides is pathognomonic for the disease. The bleeding diathesis of HPS patients, similarly to that of other types of δ-SPD, manifests with easy bruising, epistaxis, gum bleeding, menorrhagia and postsurgical bleeding. Just as the degree of hypopigmentation is not uniform in all patients, the severity of the bleeding diathesis varies substantially. It has been suggested that the bleeding risk increases in patients with low plasma vWF levels[64], although a more recent study did not confirm this association[65]. A recent report documented that major bleedings, some of which were life-threatening, occurred in 40% of the patients studied[63].

Prevalence

HPS is rare in the general population, but occurs with relatively high frequency in certain isolated groups, such as in the northwestern region of Puerto Rico, where its prevalence is 1 in 1800[66], and in an isolated village in the Swiss Alps[67].

Laboratory findings

The number of δ-granules in platelets from 7 HPS patients was markedly diminished when studied with electron microscopy, loading of the fluorescent probe mepacrine or the uranaffin reaction[36], indicating that the basic defect of HPS, at variance with that of isolated platelet δ-SPD, is a specific abnormality in organelle development which prevents the formation of an intact granule structure. In accordance with these findings, the platelet content of the δ-granule membrane protein granulophysin was shown to be very low in HPS patients[37].

The bleeding time is prolonged in most, but not all HPS patients. A variable degree of abnormalities of tests of platelet function can be observed in HPS patients as well as in patients with isolated δ-SPD. The release of α-granule proteins induced by thrombin was impaired in one patient, and was normalized by the simultaneous stimulation of platelets with ADP[68]. These findings are consistent with the recent demonstration that released ADP directly potentiates platelet secretion induced by U46619 or thrombin by interacting with the platelet $P2Y_{12}$ receptor[16,17,69]. In a group of 30 HPS patients, both plasma and

Cambridge Books Online © Cambridge University Press, 2009

platelet vWF levels tended to be slightly reduced compared to normal controls[65].

Molecular aspects

The molecular basis for HPS has only recently begun to be unravelled. HPS can arise from mutations in different genetic loci[70–72]. The Puerto Rican variant of HPS, termed HPS-1, is associated with a 16-bp duplication in exon 15 of the HPS gene, mapped to chromosome 10q23, which encodes for a cytosolic protein (HPS1p) of 79.3 kDa[73]. Another form of HPS, termed HPS-2, is characterized by mutations in the ADTB3A gene encoding the β3A subunit of the heterotetrameric protein complex, adaptor complex-3 (AP-3)[72]. The reduction in AP-3 cellular content in cells of HPS-2 patients is associated with increased routing of lysosomal proteins through the plasma membrane[72]. In contrast, cells from HPS-1 patients, which are deficient in HPS1p, display normal distribution and trafficking of lysosomal proteins[74]. It is therefore likely that HPS-2 arises from impairment of protein transport to lysosomes and related organelles, such as the platelet δ-granules, while HPS-1 arises from mechanisms that are independent of mistargeting of lysosomal membrane proteins[72,74]. Some HPS patients do not bear any detectable mutation in HPS1, ADTB3A or any other subunit gene[72]. This phenomenon, called locus heterogeneity, is also found in mouse animal models of HPS (see following section).

Animal models

Fifteen different mouse strains manifest a type of HPS (pigment dilution and platelet δ-SPD), each due to a different gene[75]. To date, three different genes have been cloned. *Pale ear* is the murine analogue of patients with HPS-1 mutations[76,77], and *pearl*[78] and *mocha*[79] have defects in AP-3. One protein subunit of AP-3, called β-3A, is mutated in the *pearl* mouse, while another protein, called δ, is mutated in the *mocha* mouse. More recently, a new mouse HPS mutation was identified, termed cappuccino (*cno*), of a gene in chromosome 5 which is not a component of the AP-3 family, confirming that there are defects in multiple pathways in the pathogenesis of HPS[80]. The mouse models of HPS show striking clinical correlations to human HPS. All have an autosomal recessive inheritance, prolonged bleeding times and suffer a bleeding diathesis.

Additional animal models of δ-SPD have been described in dogs[81], pigs[82,83] and rats[84,85].

Chediak–Higashi syndrome

Clinical and laboratory features

Chediak–Hygashi syndrome (CHS) is a rare autosomal recessive disorder characterized by variable degrees of oculocutaneous albinism, very large peroxidase-positive cytoplasmic granules in a variety of hemopoietic (neutrophils) and non-hematopoietic cells, easy bruisability due to δ-SPD, and recurrent infections, associated with neutropenia, impaired chemotaxis and bactericidal activity, and abnormal NK function[86]. Many patients may undergo an accelerated phase, characterized by non-malignant lymphoid infiltration of multiple organs. The syndrome is lethal, leading to death usually in the first decade of life.

The bleeding diathesis and the abnormalities of platelet aggregation and secretion are similar to those of other forms of δ-SPD[87,88]. The levels of the δ-granule membrane protein granulophysin are very low in CHS platelets[37], as expected in platelets lacking δ-granules[89]. The pathognomonic feature of CHS is peroxidase-positive granules that can be seen in polymorphonuclear leukocytes, as well as in megakaryocytes, neurons and other cells.

Molecular aspects

The gene responsible for CHS has been cloned[90] and mapped to the chromosome segment 1q42–44[91,92]. The gene is very large and several mutations have been described[86]. Like HPS, CHS may prove to be a genetically heterogeneous disorder, with mutations at different loci resulting in a similar phenotype.

Animal models

Among the several reported animal models for CHS[86], the *beige* mouse has been well characterized[93]. In addition to other features resembling human CHS, the *beige* mice have markedly reduced constituents of the δ-granules and prolonged bleeding times[94]. Normal bone marrow transplantation in these mice corrected the prolonged bleeding times and the platelet serotonin levels, while transplantation of *beige* mouse bone marrow into normal mice caused the development of prolonged bleeding times and low levels of δ-granule constituents[95]. The *beige* gene is located on the mouse chromosome 13, which is syntenic to human chromosome 1q42–43.

Additional animal models for the CHS have been described in cattle[96], blue foxes[97] and cats[98]. Allogeneic bone marrow transplantation successfully corrected the neutrophil migration defect and the platelet δ-SPD, but had no effect on lysosome distribution in liver and kidney cells of CHS cats[98].

Cambridge Books Online © Cambridge University Press, 2009

Hereditary thrombocytopenias

Two types of hereditary thrombocytopenia may be associated with δ-SPD: the Thrombocytopenia and absent radii syndrome (TAR)[99] and the Wiskott–Aldrich syndrome[100,101]. They are described in detail in Chapter 34 of this book.

Defects of the α-granules

α-storage pool deficiency (Grey platelet syndrome)

The first patient with this disorder was described by Raccuglia in 1971[102]. The condition owes its name to the grey appearance of the patient's platelets in peripheral blood smears as a consequence of the rarity of platelet granules. Since its first description, about 40 new cases have been reported in the literature, many of whom belonging to a single family in Japan[103]. The inheritance pattern seems to be autosomal recessive, although in a single family from Japan, it seemed to be autosomal dominant[103].

Clinical features

Affected patients have a lifelong history of mucocutaneous bleeding, which may vary from mild to moderate in severity, prolonged bleeding time, mild thrombocytopenia, abnormally large platelets and isolated reduction of the platelet α-granule content. Occasional patients may have more severe bleeding symptoms, including intracranial hemorrhage and postsurgical bleeding[104–106]. Mild to moderate myelofibrosis, which seems to be non-progressive, has been described in some patients[107] and hypothetically ascribed to the action of cytokines that are present in abnormally high concentrations in the bone marrow, as a consequence of their release by the hypogranular platelets and megakaryocytes[108]. Splenomegaly may be present[102,107,109] and splenectomy may be followed by normalization of the platelet count, but not by an amelioration of the bleeding diathesis[107]. A case report describing a GPS patient with idiopathic pulmonary fibrosis suggested the intriguing hypothesis that the abnormal megakaryocytes of GPS may have a role in the pathogenesis of this condition[106].

Laboratory findings

Gray platelets are severely and selectively deficient in soluble proteins contained in the α-granule: platelet factor 4, β-thromboglobulin, von Willebrand factor, thrombospondin, fibrinogen, albumin, etc. The extent of the deficiency is more severe for proteins that are endocytosed from plasma, such as albumin and immunoglobulins, than for proteins that are synthesized by the megakaryocytes[110–113]. In contrast to soluble proteins, the α-granule membrane proteins are normal in GPS[112,114–116], consistent with the demonstration of the presence of empty α-granules in the GPS platelets[117] and the normal production of precursors of α-granules in GPS megakaryocytes[118]. It is therefore conceivable that the basic defect in GPS is the defective targeting and packaging of endogenously synthesized proteins in platelet α-granules. This hypothesis is also consistent with the finding of increased plasma levels of β-thromboglobulin in GPS patients[119]. The targeting defect seems to be specific to the megakaryocyte cell line, as GPS patients have normal Weibel–Palade bodies, the endothelial cell storage granules equivalent to the platelet α-granules[120].

Circulating platelets are reduced in number, are relatively large, vacuolated, and contain normal numbers of mitochondria, δ-granules, peroxisomes and lysosomes but specifically lack α-granules[121]. The degree of thrombocytopenia is usually mild, although cases with platelet counts as low as 20 000 per μl have been described. Platelet aggregation studies show variable results in GPS patients. Platelet aggregation induced by ADP and adrenaline in citrated plasma were usually normal, but impaired aggregation responses induced by ADP or low concentrations of thrombin or collagen have been described in some patients[106,107,110,111,122,123]. The secretion of ^{14}C- serotonin was impaired in some patients[119], but not in others[122].

Bevers et al. reported normal platelet prothrombinase activity in three patients with GPS[56]. In contrast, a more recent study showed that the collagen plus thrombin-induced prothrombinase activity of platelets from one GPS patient was greatly impaired and was not corrected completely by the addition of exogenous factor Va[55]. The results of the latter study are consistent with the demonstration that α-granule factor V bound to the surface of platelets that had been stimulated simultaneously with thrombin and collagen, plays a unique role in generating prothrombinase activity[124].

Animal models

Rats of the Wistar Furth (WF) strain have hereditary macrothrombocytopenia, resulting from erratic subdivision of megakaryocyte cytoplasm into platelets. WF platelets have decreased levels of thrombospondin, fibrinogen and platelet factor 4, and have smaller α-granules than normal[125]. Therefore, they represent an animal model resembling GPS. The autosomal recessive pattern of inheritance of macrothrombocytes and platelet α-granule protein deficiencies suggests that a component common to formation of platelet α-granules and subdivision of megakaryocyte cytoplasm into platelets, is abnormal in WF rat megakaryocytes and platelets. WF rats have markedly prolonged bleeding times, impaired clot formation at wound sites

Cambridge Books Online © Cambridge University Press, 2009

and abnormal platelet spreading and filopodia formation after adhesion onto Formvar-coated, carbon-stabilized grids[126]. Disruption of microtubules in vivo by vincristine in normal rats caused the formation of large membrane complexes in the cytoplasm of megakaryocytes, identical to those found in megakaryocytes of human hereditary macrothrombocytopenias (including GPS) and the Wistar Furth rat[127]. Therefore, intact microtubules seem to play a major role in the organization of the megakaryocyte demarcation membrane and the stability of α-granules.

Quebec platelet disorder

The Quebec platelet disorder is an autsomal dominant qualitative platelet abnormality, characterized by severe post-traumatic bleeding complications unresponsive to platelet transfusion, abnormal proteolysis of α-granule proteins, severe deficiency of platelet factor V, deficiency of multimerin, reduced to normal platelet counts and markedly decreased platelet aggregation induced by epinephrine[128,129]. Multimerin, one of the largest proteins found in the human body, is present in platelet α-granules and in endothelial cell Weibel–Palade bodies[130–132]. It binds factor V and its activated form, factor Va. Its deficiency in patients with the Quebec platelet disorder is probably responsible for the defect in platelet factor V, which is likely to be degraded by abnormally regulated platelet proteases. Other α-granule proteins are degraded along with factor V in this disorder, including von Willebrand factor, fibrinogen, osteonectin, fibronectin, P-selectin and thrombospondin[131,133,134]. However, platelet factor 4, β-thromboglobulin, albumin, IgG, CD63 and external membrane glycoproteins are normal in these patients, indicating that there is restriction in the platelet proteins degraded[131,133,134]. In addition, electron microscopic and immunoelectron microscopy studies indicated preserved α-granular ultrastructure and normal to reduced labelling for platelet α-granule proteins, suggesting that the pathologic proteolysis of α-granule proteins is not secondary to a defect in targeting proteins to α-granules[134].

Jacobsen or Paris–Trousseau syndrome

This is rare syndrome that is associated with a mild hemorragic diathesis and is characterized by congenital thrombocytopenia, normal platelet lifespan, increased number of marrow megakaryocytes, many of which present with signs of abnormal maturation and intramedullary lysis. A fraction of the circulating platelets has giant α-granules, which are unable to release their content upon platelet stimulation with thrombin. A deletion of the distal part of one chromosome 11 [del(11)q23.3 $\rightarrow$ qter] was found in the affected patients[135,136].

Defects of the α- and δ-granules

α,δ-storage pool deficiency

Clinical and laboratory features

α,δ-storage pool deficiency is a heterogeneous congenital disorder of platelet secretion characterized by deficiencies of both α- and δ-granules[36,39]. It is important to note that blood samples should be collected in sodium citrate for measurement of platelet granule contents, because platelets from some individuals may undergo degranulation *in vitro* when blood is collected into EDTA, resembling α,δ-SPD[137]. The phenotypic heterogeneity of this disorder is illustrated by the finding that the platelet content of GMP-140 (P-selectin) was normal in three members of a family with mild α,δ-SPD, while it was approximately halved in a patient with severe α,δ-SPD[138]. Approximately 80% of platelets from the patient with severe α,δ-SPD expressed little or no GMP-140 after stimulation, whereas the remaining 20% expressed normal amounts of it. No such heterogeneity was found among platelets of the three patients with mild α,δ-SPD. Compared to δ-SPD platelets, which have a normal density, α,δ-SPD platelets show a shift to the left of the density distribution, suggesting that α-granules are a major determinant of platelet density[139].

The clinical picture and the platelet aggregation abnormalities are similar to those of patients with GPS or δ-SPD. As in GPS, the platelet prothrombinase activity in response to collagen plus thrombin was impaired in a patient with severe α,δ-SPD and was not completely corrected by added factor Va[55]. Platelet thrombus formation on everted rabbit vessel segments under various flow conditions was severely impaired[54], as well as the production of arachidonate metabolites after stimulation with arachidonate, epinephrine or collagen[44].

Animal models

Hereditary macrothrombocytopenia and prolonged bleeding times are associated with the recessive mouse pigment dilution mutant gene gunmetal (*gm/gm*). Like human α,δ-SPD, the mutant phenotype has reduced platelet levels of both δ-granule and α-granule components (serotonin, fibrinogen, platelet factor 4, von Willebrand factor)[140]. In addition, *gm/gm* platelets express two additional low molecular weight guanosine triphosphate (GTP)-binding proteins, which might be responsible for both the abnormal platelet production and the impaired organelle formation in *gm/gm* mice[139]. Further studies suggested that the gunmetal gene acts intrinsically in megakaryocytes and that an abnormality in this gene causes

Cambridge Books Online © Cambridge University Press, 2009

significant qualitative and quantitative effects on platelet production[141].

Defects of the amplification pathways of platelet activation

Defects of the arachidonate/thromboxane A_2 pathway

Impaired liberation of arachidonic acid from membrane phospholipids

Liberation of arachidonic acid from membrane phospholipids occurs under the action of phospholipase A_2 (PLA_2), a Ca^{2+}-dependent enzyme. Abnormalities of arachidonic acid liberation have been described in patients with other platelet defects, including defects of GPIIb/IIIa and procoagulant activity[142] and Hermansky–Pudlak syndrome[68]. Four patients were described with abnormal thrombin-induced liberation of ^{3}H-arachidonic acid from prelabelled platelets. TxB_2 production after stimulation with ADP or thrombin was impaired, while it was normal with arachidonic acid[143]. However, subsequent studies of one of these patients showed that his platelets contained normal amounts of PLA_2, while agonist-induced Ca^{2+} mobilization[144] and G protein activation were reduced[145].

Defects of cyclooxygenase (aspirin-like defect)

Many patients with congenital abnormalities in cyclooxygenase, the enzyme catalysing the first step in prostaglandin synthesis from arachidonate, have been described[146–155]. The platelets from these patients have the same functional defect of normal platelets that had been treated in vitro or in vivo with aspirin, which irreversibly acetylates the platelet cyclooxygenase[156,157]: impaired aggregation and secretion induced by ADP, epinephrine, collagen or arachidonic acid, normal responses to TxA_2/endoperoxides analogues, and absent platelet TxA_2 production. The actual concentration of cyclooxygenase antigen in platelet lysates, measured with an immunoassays, was found to be defective in some patients only[146,158]. It has therefore been proposed that the platelet cyclooxygenase defect can be subdivided into *type 1*, characterized by undetectable levels of the enzyme protein, and *type 2*, characterized by the presence of normal levels of a dysfunctional protein. However, before a diagnosis of *type 2* cyclooxygenase deficiency can be safely made, caution should be taken to rule out the possibility of surreptitious or inadvertent ingestion of acetylsalicylic acid, which is contained in several generic medications.

In one patient, the synthesis of both platelet TxA_2 and vessel wall PGI_2 were measured and found to be severely impaired[148]. Since that patient had a mild bleeding diathesis, the finding suggested that the contemporary abolition of TxA_2 and PGI_2 synthesis results in a hemorrhagic diathesis, rather than in a thrombotic tendency, as was previously hypothesized[159].

Defects of thromboxane synthetase

Two reports of patients with congenital defects of platelet thromboxane synthetase have been published[160,161]. Defreyn et al. described three family members of three successive generations with moderate bleeding tendency, markedly prolonged bleeding time, absent aggregation induced by arachidonic acid, and monophasic aggregation induced by ADP or epinephrine. The platelet production of TxB_2 was decreased, while that of $PGF_{2\alpha}$, PGE_2 and PGD_2 were increased. The plasma levels of the PGI_2 metabolite 6-keto $PGF_{1\alpha}$ were raised. These findings are compatible with a partial platelet thromboxane synthetase defect and reorientation of cyclic endoperoxide metabolism to increased production of the inhibitory prostaglandins PGI_2 and PGD_2, which would contribute, in conjunction with the reduced synthesis of TxA_2, to the abnormality of primary hemostasis.

Defects of the platelet thromboxane A_2 receptor

Clinical and laboratory features

In 1981, three reports of impaired platelet responses to TxA_2 in patients with bleeding disorders were published[162–164]. The platelets from these patients could synthesize TxA_2 from exogenous arachidonate, but were unable to undergo normal TxA_2-dependent aggregation and secretion in response to a variety of agonists. In one patient only, however, the stable TxA_2 mimetic U46619 was tested and found to be unable to elicit normal platelet responses[164], providing convincing evidence that his platelets had a defect at the receptor level.

In 1993, a similar patient with a mild bleeding disorder was described, whose platelets did not undergo shape change, aggregation and secretion in response to the synthetic TxA_2 mimetic STA_2[165]. Binding studies of radiolabelled TxA_2 agonists and antagonists revealed that the patient platelets had a normal number of TxA_2 binding sites and normal equilibrium dissociation rate constants. Despite the normal number of TxA_2 receptors, the TxA_2-induced IP3 formation, Ca^{2+} mobilization and GTPase activity were normal, suggesting that the abnormality of these platelets was impaired coupling between TxA_2 receptor, G protein and PLC. The platelet aggregation and secretion responses to several agonists were impaired. A similar

Cambridge Books Online © Cambridge University Press, 2009

patient, who was also affected by polycythemia vera, had previously been described by Ushikubi et al.[166].

Molecular aspects
These two last patients were subsequently found to have an Arg60 to Leu mutation in the first cytoplasmic loop of the TxA_2 receptor[167], affecting both isoforms of the receptor[168,169]. The mutant receptor expressed in Chinese hamster ovary cells showed decreased agonist-induced second messenger formation despite its normal ligand binding affinities. The mutation was found exclusively in the affected members of the two unrelated families and was inherited as an autosomal dominant trait. Although the heterozygous patients did not differ from the homozygous patients in terms of aggregation and secretion responses of platelets to TxA_2, subsequent studies showed that in heterozygous patients, the mutant TxA_2 receptor suppresses the wild-type receptor-mediated platelet aggregation and secretion by a mechanism independent of inhibition of PLC activation[170,171].

Animal models
Most dogs have blood platelets that do not aggregate or secrete in response to TxA_2, although they form normal amounts of it[172–175]. TxA_2 unresponsive dog platelets have impaired TxA_2 receptor-G protein coupling, and abnormal binding parameters of the TxA_2 analogue BOP; all platelet abnormalities could be corrected by platelet treatment with epinephrine[176].

In contrast to dog, in whom the platelet insensitivity to TxA_2 is the rule, normal mice have TxA_2 platelet receptors. TxA_2 knockout mice have a mild bleeding disorder and altered vascular responses to TxA_2 and arachidonate[177].

Congenital defects of the ADP pathway (see Fig. 43.2)

Platelets possess at least three different purinergic receptors[178–183]: the $P2X_1$ ionotropic receptor responsible for rapid influx of ionized calcium into the cytosol; the $P2Y_1$ metabotropic receptor responsible for mobilization of ionized calcium from internal stores which initiates ADP-induced aggregation, and a third receptor coupled to adenylyl cyclase inhibition, essential for the full aggregation response to ADP, which has recently been identified[184,185] and named $P2Y_{12}$[184]. Although $P2X_1$ may be important for normal hemostasis, since a dominant negative mutation in its gene has been reported in a patient with a severe bleeding disorder[186], recent data have provided convincing evidence that it is activated by ATP, rather than ADP[187]. Therefore, platelets seem to have only two purinoceptors for ADP: $P2Y_1$ and $P2Y_{12}$. Only patients with congenital

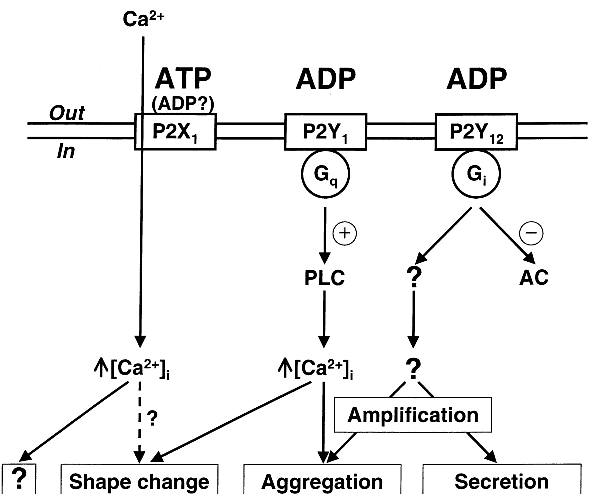


Fig. 43.2. Effects of platelet purinoceptors on platelet function. The role of $P2X_1$ is unclear: although many studies failed to find any effect of $P2X_1$ on platelet function, a recent report showed that it mediates the platelet shape change triggered by the ATP analogue α,β-methylene ATP[214]. ADP, adenosine diphosphate; ATP, adenosine triphosphate; $[Ca^{2+}]_i$, concentration of cytoplasmic ionized calcium; AC, adenylyl cyclase; PLC, phospholipase C; cAMP, cyclic adenosine monophosphate.

abnormalities of $P2Y_{12}$ have been described so far[183]. The platelet function abnormality of patients who are heterozygous for the defect of $P2Y_{12}$ receptors is typical of disorders of platelet secretion[69], since $P2Y_{12}$ is the mediator of the described potentiating effect of ADP on platelet secretion[16,17,183]. Patients who are homozygous for $P2Y_{12}$ defect, in addition to a secretion defect, have a severe impairment of platelet aggregation induced by ADP.

Congenital defects of the $P2Y_{12}$ receptor for ADP

In 1992, the first patient with a selective defect of platelet responses to ADP was described[188]. He is a man of Caucasian origin who had a lifelong history of excessive bleeding, prolonged bleeding time and an abnormality of platelet aggregation that was similar to that observed in patients with defects of platelet secretion, except that the aggregation response to ADP was severely impaired. This platelet aggregation profile is compatible with a selective impairment of responses to ADP, which also affects the full aggregation response to release-inducing agonists, as a consequence of the loss of the potentiating effect of released ADP on platelet aggregation. Other abnormalities of platelet function found in this patient were: (i) no inhibition by ADP of PGE_1-stimulated platelet adenylate cyclase, but normal inhibition by epinephrine; (ii) normal shape change and normal-to-mildly reduced mobilization

Cambridge Books Online © Cambridge University Press, 2009

of cytoplasmic ionized calcium induced by ADP; (iii) presence of about 30% of the normal number of platelet binding sites for $[^3H]ADP$[188] or $[^{33}P]2MeSADP$[189]. Three additional patients, one male[190] and two females[69] with very similar characteristics were subsequently described; in one of them it was shown that the ADP receptor pathway that is defective in this condition is linked to a defective tyrosine phosphorylation response[191]. The many similarities among the patients suggest that they are affected by the same type of disorder of platelet function[192], associated with defective interaction between ADP and the recently cloned $P2Y_{12}$ receptor. The deletion of two nucleotides causing a shift in the reading frame of the coding region of one allele and repression of the expression of the wild-type allele were described in one of the patients[190] with $P2Y_{12}$ deficiency[184]. The defect is probably inherited as an autosomal recessive trait, because all patients so far described were born from consanguineous parents.

The heterozygous son of one of these patients bound intermediate levels of 2MeS–ADP[69]. His bleeding time was mildly prolonged (13 min) and his platelets underwent a normal first wave of aggregation after stimulation with ADP, but did not secrete normal amounts of ATP after stimulation with different agonists. The secretion defect of his platelets could not be ascribed to impaired production of thromboxane A_2 or low concentrations of platelet granule contents, which were normal and was therefore attributed to the partial defect in the potentiation of platelet secretion mediated by $P2Y_{12}$. His phenotype is therefore similar to that of some previously described patients with secretion defects of unknown cause, who proved to be partially deficient in $P2Y_{12}$ receptors[16].

Miscellaneous abnormalities of platelet secretion (primary secretion defects)

Definition

The term primary secretion defect was probably used for the first time by Weiss, to indicate all those ill-defined abnormalities of platelet secretion not associated with platelet granules deficiencies[193]. The term was later used to indicate the platelet secretion defects not associated to platelet granule with deficiencies and abnormalities of the arachidonate pathway[16,69], or, more generally, all the abnormalities of platelet function associated with defects of signal transduction[194,195]. With the progression of our knowledge in the platelet pathophysiology, this heterogeneous group, which lumps together the majority of patients with congenital disorders of platelet function[194], will become progressively smaller, losing those patients with better defined biochemical abnormalities responsible for their platelet secretion defect. An example is given by patients with heterozygous $P2Y_{12}$ deficiency who were included in this group of disorders until their biochemical abnormality was identified[16,69,183].

Only those abnormalities of platelet secretion that exhibit normal primary waves of aggregation induced by ADP, epinephrine or PAF will be described here.

Clinical and laboratory features

In 1981, Wu et al. described a large family with a hereditary bleeding disorder associated with defective platelet secretion, despite normal platelet granules contents and normal TxA_2 production[196]. Although the platelets of these patients might have had an abnormality of TxA_2 receptors, because they did not respond to a PGH_2 analogue, their biochemical abnormality has not been further elucidated. Hardisty et al. later reported a patient with similar platelet abnormalities, including the lack of reponse to PGH_2 and TxA_2[197]. Of interest are some abnormalities of platelet secretion described in patients with psychiatric disorders, such as the attention deficit disorder[198] and conduct disorder[199]: these reports emphasize the role of platelets as a model for neurons in functional disorders. In 1989, Rao et al. described a 42-year-old white woman and her 23-year-old son who suffered from a mild hemorrhagic diathesis, whose platelets underwent abnormal secretion and aggregation induced by ADP, epinephrine, PAF, arachidonic acid and ionophore A23187[200]. In both patients, the platelet ADP and ATP contents and TxA_2 synthesis were normal, while the concentration of cytoplasmic Ca^{2+} $[Ca^{2+}]i$ in resting platelets, peak $[Ca^{2+}]i$ concentrations stimulated by ADP, PAF, collagen, the prostaglandin endoperoxides analogue U46619 and thrombin were impaired. Subsequent studies indicated that, upon platelet activation, the formation of inositol 1,4,5-trisphosphate (In1,4,5–P_3) and diacylglycerol, and the phosphorylation of pleckstrin were abnormal[201]. These data suggested that the patient platelets had a defect in phospholipase C (PLC) activation. This hypothesis was confirmed by the finding that platelets from one of these patients had a selective decrease in one of the seven PLC isoforms present in platelets, PLC-β2, suggesting that this isoenzyme may have an important role in platelet activation[202].

Generally included in the group of Primary Secretion Defects are patients with abnormal secretion, normal platelet granules and TxB_2 formation, but decreased initial rate and extent of aggregation to one or more weak

Cambridge Books Online © Cambridge University Press, 2009

Table 43.3. Therapeutic options for congenital defects of platelet secretion

Desmopressin (DDAVP)
0.3 μg per kilogram b.w., intravenously or subcutaneously
300 μg (adults) or 150 μg (children), intanasally

Antifibrinolytic agents
Tranexamic acid: 15–25 mg per kilogram b.w every 8 hours, orally or intravenously
ε-aminocaproic acid: 50–60 mg per kilogram b.w every 4 hours, orally or intravenously

Transfusion of platelet concentrates
Required only for the control of severe bleeding episodes, which are very rare in patients with platelet secretion defects.

agonists[203]. These defects will be described in detail in the following chapter of this book.

Therapy (see Table 43.3)

The mainstays of therapy of patients with congenital disorders of platelet secretion are platelet transfusions, the vasopressin analogue 1-deamino-8–D-arginine vasopressin (desmopressin, or DDVAP) and antifibrinolytic agents.

Bleeding complications in patients with disorders of platelet secretion are rarely severe enough to require the transfusion of platelet concentrates, which are effective but are expensive and carry the risks that are associated with the use of blood products.

Desmopressin, which causes the increase in plasma vWF and FVIII levels, has been shown to be effective in shortening the prolonged bleeding times of most patients with disorders of platelet secretion, including patients with Hermansky–Pudlak syndrome[204] and other platelet function abnormalities, including Bernard–Soulier syndrome and drug-induced platelet dysfunction[52,205–210]. Since the bleeding time response to DDAVP infusions varies very much among subjects and tends to be rather consistent after repeated infusions, a test dose of DDAVP should be administered in order to test the degree of responsiveness to the drug of each patient. Although the effect of desmopressin on the bleeding times of patients with platelet function disorders is well established, its efficacy in reducing the incidence and severity of bleeding complications in these patients is based on anecdotal reports only[210–212]. Despite the absence of controlled clinical trials documenting its efficacy, desmopressin can be recommended for the prophylaxis and treatment of bleeding episodes in patients with platelet function disorders, in consideration of the absence of serious side effects associated with its infusion[205,211]. The mechanisms by which desmopressin shortens the prolonged bleeding times of patients with normal plasma vWF levels are not well understood: both mechanisms that are dependent[28,52] and independent[213] on the induced increase in plasma vWF levels have been hypothesized.

Other approaches used to reduce the bleeding episodes in patients with platelet secretion defects include the administration of antifibrinolytic agents, such as ε-aminocaproic acid and tranexamic acid[205,211]. Tranexamic acid reduces menstrual bleeding by 40 to 50 percent. It should be used only when organic lesions in the uterus have been excluded, at the oral doses of 10 to 15 mg per kilogram of body weight every 8 hours, from the onset until the arrest of menstrual bleeding. Antifibrinolytic agents may also be useful in the control of oral bleeding in these patients.

References

1 Nishibori M, Cham B, McNicol A, Shalev A, Jain N, Gerrard JM. The protein CD63 is in platelet dense granules, is deficient in a patient with Hermanski–Pudlak syndrome, and appears identical to granulophysin. *J Clin Invest* 1993; 91: 1775–1782.
2 Israels SJ, Gerrard JM, Jacques YV et al. Platelet dense granule membranes contain both granulophysin and P-selectin (GMP-140). *Blood* 1992; 80: 143–152.
3 Youssefian T, Masse JM, Rendu F, Guichard J, Cramer EM. Platelet and megakaryocyte dense granules contain glycoproteins Ib and IIb–IIIa. *Blood* 1997; 89: 4047–4057.
4 George JN. Platelet immunoglobulin G: its significance for the evaluation of thrombocytopenia and for understanding the origin of alpha-granule proteins. *Blood* 1990; 76: 859–870.
5 Cramer EM. Platelets and medgakaryocytes: anatomy and structural organization. In Colman RW, Hirsh J, Marder VJ, Clowes AW, George JN, eds. *Hemostasis and Thrombosis. Basic Principles and Clinical Practice.* Philadelphia, PA: J.B. Lippincott Williams and Wilkins; 2001: 411–428.
6 Leung LL. Role of thrombospondin in platelet aggregation. *J Clin Invest* 1984; 74: 1764–1772.
7 Cattaneo M, Canciani MT, Lecchi A et al. Released adenosine diphosphate stabilizes thrombin-induced human platelet aggregates. *Blood* 1990; 75: 1081–1086.
8 Chen D, Lemons PP, Schraw T, Whiteheart SW. Molecular mechanisms of platelet exocytosis: role of SNAP-23 and syntaxin 2 and 4 in lysosome release. *Blood* 2000; 96: 1782–1788.
9 Ciferri W, Emiliani C, Guglielmini G, Orlacchio A, Nenci GG, Gresele P. Platelets release their lysosomal content in vivo in humans upon activation. *Thromb Haemost* 2000; 83, 157–164.

Cambridge Books Online © Cambridge University Press, 2009

10 Polasek J. Lysosomal concept of platelet secretion – revisited. *Eur J Haematol* 1989; 50 (Suppl.): 3–24.
11 Si-Tahar M, Renesto P, Falet H, Rendu F, Chignard M. The phospholipase C/protein kinase C pathway is involved in cathepsin G-induced human platelet activation: comparison with thrombin. *Biochem J* 1996; 313: 401–408.
12 Lemons PP, Chen D and Whiteheart S W. Molecular mechanisms of platelet exocytosis: requirements for alpha-granule release. *Biochem Biophys Res Commun* 2000; 267: 875–880.
13 Reed GL, Fitzgerald ML, Polgar J. Molecular mechanisms of platelet exocytosis: insights into the 'secrete' life of thrombocytes. *Blood* 2000; 96: 3334–3342.
14 Mustard JF, Perry DW, Kinlough-Rathbone RL, Packham MA. Factors responsible for ADP-induced release reaction of human platelets. *Am J Physiol* 1975; 228: 1757–1765.
15 Falcon CR, Cattaneo M, Ghidoni A, Mannucci PM. The *in vitro* production of thromboxane B2 by platelets of diabetic patients is normal at physiological concentrations of ionised calcium. *Thromb Haemost* 1993; 70: 389–392.
16 Cattaneo M, Lombardi R, Zighetti ML et al. Deficiency of [33P]2MeS-ADP binding sites on platelets with secretion defect, normal granule stores and normal thromboxane A2 production. Evidence that ADP potentiates platelet secretion independently of the formation of large platelet aggregates and thromboxane A2 production. *Thromb Haemost* 1997; 77: 986–990.
17 Cattaneo M, Lecchi A. ADP potentiates platelet dense granule secretion induced by U46619 or TRAP through its interaction with the P2cyc receptor. *Haematologica* 2000; 85 (supplement): 86.
18 Packham MA, Kinlough-Rathbone RL, Mustard JF. Thromboxane A2 causes feedback amplification on close contact of human platelets in media with a low concentration of ionized calcium. *Blood* 1987; 70: 647–651.
19 Cattaneo M, Lecchi A, Canciani MT. Conditions influencing the in vitro response of human platelets to thrombin and collagen. Effects of $[Ca^{2+}]_o$ and aggregation. *Blood* 1987; 70 (Suppl): 349 (abstract).
20 Rao AK, Willis J, Hassell B, Dangelmaier C, Holmsen H, Smith JB. Platelet-activating factor is a weak platelet agonist: evidence from normal human platelets with congenital secretion defects. *Am J Hematol* 1984; 17: 153–165.
21 WeissHJ, Chervenick PA, Zalusky R, Factor A. A familial defect in platelet function associated with impaired release of adenosine diphosphate. *N Engl J Med* 1969; 281: 1264–1270.
22 Holmsen H, Weiss HJ. Hereditary defect in the platelet release reaction caused by a deficiency in the storage pool of platelet adenine nucleotides. *Br J Haematol* 1970; 19: 643: 649.
23 Holmsen H, Weiss HJ. Further evidence for a deficient storage pool of adenine nucleotides in platelets from some patients with thrombocytopenia – 'storage pool disease'. *Blood* 1972; 39: 197–209.
24 Nieuwenhuis HK, Akkerman JW, Sixma JJ. Patients with a prolonged bleeding time and normal aggregation tests may have storage pool deficiency: studies on 106 patients. *Blood* 1987; 70: 620–623.
25 Rao AK. Congenital disorders of platelet secretion and signal transduction. In Colman RW, Hirsh J, Marder VJ, Clowes AW, George JN, eds. *Hemostasis and Thrombosis. Basic Principles and Clinical Practice*. Philadelphia, PA: J.B. Lippincott Williams and Wilkins; 2001: 893–904.
26 Tsementzis SA, Marsh J. Spontaneous subarachnoid hemorrhage occurring in association with a platelet function disorder. *Neurosurg Rev* 1991; 14: 57–59.
27 Akkerman JW, Nieuwenhuis HK, Mommersteeg-Leautaud ME, Gorter G, Sixma JJ. ATP-ADP compartmentation in storage pool deficient platelets: correlation between granule-bound ADP and the bleeding time. *Br J Haematol* 1983; 55: 135–43.
28 Cattaneo M, Lecchi A, Agati B, Lombardi R, Zighetti ML. Evaluation of platelet function with the PFA-100 system in patients with congenital defects of platelet secretion. *Thromb Res* 1999; 96: 213–217.
29 Gerrard JM, Israels ED, Bishop AJ, Schroeder ML, Beattie LL, McNicol A, Israels SJ, Walz D, Greenberg AH, Ray M. Inherited platelet storage pool deficiency associated with a high incidence of acute myeloid leukemia. *Br J Haematol* 1991; 79: 246–255.
30 Gerrard JM, McNicol A. Platelet storage pool deficiency, leukemia, and myelodysplastic syndromes. *Leuk Lymphoma* 1992; 8: 277–281.
31 Herve P, Drouet L, Dosquet C, Launay JM, Rain B, Simonneau G, Caen J, Duroux P. Primary pulmonary hypertension in a patient with a familial platelet storage pool disease: role of serotonin. *Am J Med* 1990; 89: 117–120.
32 Kentera D, Susic D, Veljkovic V, Tucakovic G, Koko V. Pulmonary artery pressure in rats with hereditary platelet function defect. *Respiration* 1988; 54: 110–4.
33 Richards JG, Da Prada M. Uranaffin reaction: a new cytochemical technique for the localization of adenine nucleotides in organelles storing biogenic amines. *J Histochem Cytochem* 1977; 25: 1322–1326.
34 Lorez HP, Da Prada M, Rendu F, Pletscher A. Mepacrine, a tool for investigating the 5-hydroxytryptamine organelles of blood platelets by fluorescence microscopy. *J Lab Clin Med* 1977; 89: 200–206.
35 White JG. Use of the electron microscope for diagnosis of platelet disorders. *Semin Thromb Haemost* 1998; 24: 163–168.
36 Weiss HJ, Lages B, Vicic W, Tsung LY, White JG. Heterogeneous abnormalities of platelet dense granule ultrastructure in 20 patients with congenital storage pool deficiency. *Br J Haematol* 1993; 83: 282–295.
37 Shalev A, Michaud G, Israels SJ, McNicol A, Singhroy S, McMillan EM, White JG, Witkop CJ, Nichols WL, Greenberg AH. Quantification of a novel dense granule protein (granulophysin) in platelets of patients with dense granule storage pool deficiency. *Blood* 1992; 80: 1231–1237.
38 McNicol A, Israels SJ, Robertson C, Gerrard JM. The empty sack syndrome: a platelet storage pool deficiency associated with empty dense granules. *Br J Haematol* 1994; 86: 574–582.
39 Weiss HJ, Witte LD, Kaplan KL, Lages B, Chernoff A, Nossel HL, Goodman DeWitt S, Baumgartner HR. Heterogeneity

Cambridge Books Online © Cambridge University Press, 2009

in storage pool deficiency: Studies on granule-bound substances in 18 patients including variants deficient in α-granules, platelet factor 4, β-thromboglobulin, and platelet-derived growth factor. *Blood* 1979; 54: 1296–1319.

40 Lages B, Scrutton MC, Holmsen H, Day JJ, Weiss HJ. Metal ion content of gel-filtered platelets from patients with storage pool disease. *Blood* 1975; 46: 119–130.

41 Holmsen H, Weiss HJ. Secretable storage pools in platelets. *Ann Rev Med* 1979; 30: 119–134.

42 Pareti FI, Day HJ, Mills DCB. Nucleotide and serotonin metabolism in platelets with defective secondary aggregation. *Blood* 1974; 44: 789–800.

43 Ingerman CM, Smith JB, Shapiro S, Sedar A, Silver MJ. Hereditary abnormality of platelet aggregation attributable to nucleotide storage pool deficiency. *Blood* 1978; 52: 332–344.

44 Weiss HJ, Lages B. Platelet malondialdehyde production and aggregation responses induced by arachidonate, prostaglandin-G2, collagen, and epinephrine in 12 patients with storage pool deficiency. *Blood* 1981; 58: 27–33.

45 Weiss HJ, Willis AS, Kuhn D, Brand H. Prostaglandin E2 potentiation of platelet aggregation induced by LASS endoperoxide: absent in storage pool disease, normal after aspirin ingestion. *Br J Haematol* 1976; 32: 257–272.

46 Malmsten C, Kindahl H, Samuelsson B, Levy-Toledano S, Tobelem G, Caen JP. Thromboxane synthesis and the platelet release reaction in Bernard–Soulier syndrome, Thrombasthenia Glanzmann and Hermansky–Pudlak syndrome. *Br J Haematol* 1977; 35: 511–520.

47 Minkes MS, Joist JH, Needleman P. Arachidonic acid-induced platelet aggregation independent of ADP-release in a patient with a bleeding disorder due to platelet storage pool disease. *Thromb Res* 1979; 15: 169–179.

48 Lages B, Weiss HJ. Biphasic aggregation responses to ADP and epinephrine in some storage pool deficient platelets: relationship to the role of endogenous ADP in platelet aggregation and secretion. *Thromb Haemost* 1980; 18: 147–153.

49 Israels SJ, McNicol A, Robertson C, Gerrard JM. Platelet storage pool deficiency: diagnosis in patients with prolonged bleeding times and normal platelet aggregation. *Br J Haematol* 1990; 75: 118–121.

50 Holmsen H, Setkowsky CA, Lages B, Day HJ, Weiss HJ, Scrutton MC. Content and thrombin-induced release of acid hydrolases in gel-filtered platelets from patients with storage pool disease. *Blood* 1975; 46: 131–142.

51 Lages B, Dangelmaier CA, Holmsen H, Weiss HJ. Specific correction of impaired acid hydrolase secretion in storage pool-deficient platelets by adenosine diphosphate. *J Clin Invest* 1988; 81: 1865–1872.

52 Cattaneo M, Pareti FI, Zighetti ML, Lecchi A, Lombardi R, Mannucci PM. Platelet aggregation at high shear is impaired in patients with congenital defects of platelet secretion and is corrected by DDAVP: correlation with the bleeding time. *J Lab Clin Med* 1995; 125: 540–547.

53 Weiss HJ, Tschopp TB, Baumgartner HR. Impaired interaction (adhesion-aggregation) of platelets with the subendothelium in storage-pool disease and after aspirin ingestion. A comparison with von Willebrand's disease. *N Engl J Med* 1975; 293: 619–623.

54 Weiss HJ, Turitto VT, Baumgartner HR. Platelet adhesion and thrombus formation on subendothelium in platelets deficient in glycoproteins IIb–IIIa, Ib, and storage granules. *Blood* 1986; 67: 322–330.

55 Weiss HJ, Lages B. Platelet prothrombinase activity and intracellular calcium responses in patients with storage pool deficiency, glycoprotein IIb–IIIa deficiency, or impaired platelet coagulant activity – A comparison with Scott Syndrome. *Blood* 1997; 89: 1599–1611.

56 Bevers EM, Comfurius P, Nieuwenhuis HK et al. Platelet prothrombin converting activity in hereditary disorders of platelet function. *Br J Haematol* 1986; 63: 336–345.

57 Gordon N, Thom J, Cole C, Baker R. Rapid detection of hereditary and acquired platelet storage pool deficiency by flow cytometry. *Br J Haematol* 1995; 89: 117–123.

58 Wall JE, Buijs-Wilts M, Arnold JT et al. A flow cytometric assay using mepacrine for study of uptake and release of platelet dense granule contents. *Br J Haematol* 1995; 89: 380–385.

59 Spritz RA. Multi-organellar disorders of pigmentation – tied up in traffic. *Clin Genet* 1999; 55: 309–317.

60 Garay SM, Gardella JE, Fazzini EP, Goldring RM. Hermansky–Pudlak syndrome. Pulmonary manifestations of a ceroid storage disorder. *Am J Med* 1979; 66: 737–747.

61 Brantly M, Avila NA, Shotelersuk V, Lucero C, Huizing M, Gahl WA. Pulmonary function and high-resolution CT findings in patients with an inherited form of pulmonary fibrosis, Hermansky–Pudlak syndrome, due to mutations in HPS-1. *Chest* 2000; 117: 129–136.

62 Schinella RA, Greco MA, Cobert BL, Denmark LW, Cox RP. Hermansky–Pudlak syndrome with granulomatous colitis. *Ann Intern Med* 1980; 92: 20–23.

63 Gahl WA, Brantly M, Kaiser-Kupfer I et al. Genetic defects and clinical characteristics of patients with a form of oculocutaneous albinism (Hermansky–Pudlak syndrome). *N Engl J Med* 1998; 338: 1258–1264.

64 Witkop CJ, Bowie EJW, Krumwiede MD, Swanson JL, Plumhoff EA, White JG. Synergistic effect of storage pool deficient platelets and low plasma von Willebrand factor on the severity of the hemorrhagic diathesis in Hermansky–Pudlak syndrome. *Am J Haematol* 1993; 46: 256–259.

65 McKeown LP, Hansmann KE, Wilson O et al. Platelet von Willebrand factor in Hermansky–Pudlak syndrome. *Am J Hematol* 1998; 59: 115–120.

66 Witkop CJ, Nunez Babcock M, Rao GH et al. Albinism and Hermansky–Pudlak syndrome in Puerto Rico. *Bol Asoc Med P R* 1990; 82: 333–339.

67 Lattion F, Schneider P, Da Prada M et al. Hermansky–Pudlak syndrome in a Valais village. *Helv Paediatr Acta* 1983; 38: 495–512.

68 Rendu F, Maclouf J, Launay JM et al. Hermansky–Pudlak platelets: further studies on release reaction and protein phosphorylations. *Am J Hematol* 1987; 25: 165–174.

69 Cattaneo M, Lecchi A, Lombardi R, Gachet C, Zighetti ML.

Cambridge Books Online © Cambridge University Press, 2009

Platelets from a patient heterozygous for the defect of P2cyc receptors for ADP have a secretion defect despite normal thromboxane A2 production and normal granule stores. Further evidence that some cases of platelet 'Primary Secretion Defect' are heterozygous for a defect of P2cyc receptors. *Arterioscler Thromb Vasc Biol* 2000; 20: e101–e106.
70 Hazelwood S, Shotelersuk V, Wildenberg SC et al. Evidence for locus heterogeneity in Puerto Ricans with Hermansky-Pudlak syndrome. *Am J Hum Genet* 1997; 61: 1088–1094.
71 Oh J, Ala-Mello S, Amato D et al. Mutation analysis of patients with Hermansky–Pudlak syndrome: a frameshift hot spot in the HPS gene and apparent locus heterogeneity. *Am J Hum Genet* 1998; 62: 593–598.
72 Dell'Angelica EC, Shoteleresuk V, Aguilar RC, Gahl WA, Bonifacino JS. Altered trafficking of lysosomal proteins in Hermansky–Pudlak syndrome due to mutations in the beta3A subunit of the AP-3 adaptor. *Mol Cell* 1999; 3: 11–21.
73 Oh J, Bailin T, Kazuyoshi F et al. Positional cloning of a gene for Hermansky-Pudlak syndrome, a disorder of cytoplasmic organelles. *Nat Genet* 1996; 14: 300–306.
74 Dell'Angelica EC, Aguilar RC, Wolins N, Hazelwood S, Gahl WA, Bonifacino JS. Molecular characterization of the protein encoded by the Hermansky–Pudlak syndrome type 1 gene. *J Biol Chem* 2000; 275: 1300–1306.
75 Swank RT, Novak EK, McGarry MP, Rusiniak ME, Feng L. Mouse models of Hermansky–Pudlak syndrome: a review. *Pigment Cell Res* 1998 1998; 11: 60–80.
76 Feng GH, Bailin T, Oh J, Spritz RA. Mouse pale ear (ep) is homologous to human Hermansky–Pudlak syndrome and contains a rare 'AT-AC' intron. *Hum Mol Genet* 1997; 6: 793–797.
77 Gardner JM, Wildenberg SC, Keiper NM et al. The mouse pale ear (ep) mutation is the homologue of human Hermansky–Pudlak syndrome. *Proc Natl Acad Sci USA* 1997; 94: 9238–9243.
78 Feng L, Seymour AB, Jiang S et al. The beta3A subunit gene (Ap3b1) of the AP-3 adaptor complex is altered in the mouse hypopigmentation mutant pearl, a model for Hermansky–Pudlak syndrome and night blindness. *Hum Mol Genet* 1999; 8: 323–330.
79 Kantheti P, Qiao X, Diaz ME et al. Mutation in AP-3 delta in the mocha mouse links endosomal transport to storage deficiency in platelets, melanosomes, and synaptic vesicles. *Neuron* 1998; 21: 111–122.
80 Gwynn B, Ciciotte SL, Hunter SJ et al. Defects in the cappuccino (cno) gene on mouse chromosome 5 and human 4p cause Hermansky–Pudlak syndrome by an AP-3-independent mechanism. *Blood* 2000; 96: 4227–4235.
81 Callan MB, Bennett JS, Phillips DK et al. Inherited platelet delta-storage pool disease in dogs causing severe bleeding: an animal model for a specific ADP deficiency. *Thromb Haemost* 1995; 74: 949–953.
82 Daniels TM, Fass DN, White JG, Bowie EJ. Platelet storage pool deficiency in pigs. *Blood* 1986; 67: 1043–1047.
83 Radvanyi-Hofmann H, Roussi J, Launay JM et al. Characterization of a thrombopathy (type delta storage pool disease) affecting a pig colony. *Nouv Rev Fr Hematol* 1992; 34: 133–140.
84 Raymond SL, Dodds WJ. Characterization of the fawn-hooded rat as a model for hemostatic studies. *Thromb Diath Haemorrh* 1975; 33: 361–369.
85 Tschopp B, Weiss HJ. Decreased ATP, ADP and serotonin in young platelets of fawn-hooded rats with storage pool disease. *Thromb Diath Haemorrh* 1974; 32: 670–677.
86 Introne W, Boissy RE, Gahl WA. Clinical, molecular, and cell biological aspects of Chediak-Higashi Syndrome. *Mol Genet Metab* 1999; 68: 283–303.
87 Buchanan GR, Handin RI. Platelet function in the Chediak–Higashi syndrome. *Blood* 1976; 47: 941–948.
88 Apitz-Castro R, Cruz MR, Ledezma E et al. The storage pool deficiency in platelets from humans with the Chediak–Higashi syndrome: study of six patients. *Br J Haematol* 1985; 59: 471–483.
89 Rendu F, Breton-Gorius J, Lebret M et al. Evidence that abnormal platelet functions in human Chediak-Higashi syndrome are the result of a lack of dense bodies. *Am J Pathol* 1983; 111: 307–314.
90 Nagle DL, Karim MA, Woolf EA et al. Identification and mutation analysis of the complete gene for Chediak–Higashi syndrome. *Nat Genet* 1996; 14: 307–311.
91 Barrat FJ, Auloge L, Pastural E et al. Genetic and physical mapping of the Chediak–Higashi syndrome on chromosome 1q42–43. *Am J Hum Genet* 1996; 59: 625–632.
92 Fukai K, Oh J, Karim MA et al. Homozygosity mapping of the gene for Chediak–Higashi syndrome to chromosome 1q42–44 in a segment of conserved synteny that includes the mouse *beige* locus (bg). *Am J Hum Genet* 1996; 59: 620–624.
93 Lutzner MA, Lowrie CT, Jordan HW. Giant granules in leukocytes of the beige mouse. *J Hered* 1967; 58: 299–300.
94 Novak EK, Hui S-W, Swank RT. Platelet storage pool deficiency in mouse pigment mutations associated with seven distinct loci. *Blood* 1985; 63: 536–544.
95 Novak EK, McGarry MP, Swank RT. Correction of symptoms of platelet storage pool deficiency in animal models for Chediak–Higashi syndrome and Hermansky–Pudlak syndrome. *Blood* 1985; 66: 1196–1201.
96 Menard M, Meyers KM. Storage pool deficiency in cattle with the Cediak–Higashi syndrome results from an absence of dense granule precursors in their megakaryocytes. *Blood* 1988; 72: 1726–1734.
97 Sjaastad OV, Blom AK, Stormorken H, Nes N. Adenine nucleotides, serotonin, and aggregation properties of platelets of blue foxes (*Alopex lagopus*) with the Chediak–Higashi syndrome. *Am J Med Genet* 1990; 35: 373–378.
98 Colgan SP, Hull-Thrall MA, Gasper PW et al. Restoration of neutrophil and platelet function in feline Chediak–Higashi syndrome by bone marrow transplantation. *Bone Marrow Transpl* 1991; 7: 365–374.
99 Day HJ, Holmsen H. Platelet adenine nucleotide 'storage pool deficiency' in thrombocytopenic absent radii syndrome. *JAMA* 1972; 28: 1053–1054.
100 Grottum KA, Hovig T, Holmsen H, Abrahamsen AF, Jeremic M,

Cambridge Books Online © Cambridge University Press, 2009

Seip M. Wiskott–Aldrich syndrome: qualitative platelet defects and short platelet survival. *Br J Haematol* 1969; 17: 373–388.
101 Remold-O'Donnel E, Rosen FS, Kenney DM. Defects in Wiskott–Aldrich syndrome blood cells. *Blood* 1996; 87: 2621–2631.
102 Raccuglia G. Gray platelet syndrome: a variety of qualitative platelet disorder. *Am J Med* 1971; 51: 818–828.
103 Mori K, Suzuki S, Sugai K. Electron microscopic and functional studies on platelets in gray platelet syndrome. *Tohoku J Exp Med* 1984; 143: 261–287.
104 Gootenberg JE, Buchanan GR, Holtkamp CA, Casey CS. X Severe hemorrhage in a patient with gray platelet syndrome. *J Pediatr* 1984; 109: 1017–1019.
105 Berrebi A, Klepfish A, Varon D et al. Gray platelet syndrome in the elderly. *Am J Haematol* 1988; 28: 270–272.
106 Facon T, Goudemand J, Caron C et al. Simultaneous occurrence of grey platelet syndrome and idiopathic pulmonary fibrosis: a role for abnormal megakaryocytes in the pathogenesis of pulmonary fibrosis? *Br J Haematol* 1990; 74: 542–543.
107 Jantunen E, Hanninen A, Naukkarinen A, Vornanen M, Lahtinen R. Gray platelet syndrome with splenomegaly and signs of extramedullary hematopoiesis: a case report with review of the literature. *Am J Haematol* 1994; 46: 218–224.
108 Caen JP, Deschamps JF, Bodevin E, Bryckaert MC, Dupuy E, Wasteson A. Megakaryocytes and myelofibrosis in gray platelet syndrome. *Nouv Rev Fr Hematol* 1987; 29: 109–114.
109 Baruch D, Lindhout T, Dupuy E, Caen JP. Thrombin-induced platelet fractor Va formation in patients with a gray platelet syndrome. *Thromb Haemost* 1987; 58: 768–771.
110 Gerrard JM, Phillips DR, Rao GH et al. Biochemical studies of two patients with the gray platelet syndrome. Selective deficiency of platelet alpha granules. *J Clin Invest* 1980; 66: 102–109.
111 Nurden AT, Kunicki TJ, Dupuis D, Soria C, Caen JP. Specific protein and glycoprotein deficiencies in platelets isolated from two patients with the gray platelet syndrome. *Blood* 1982; 59: 709–718.
112 Rosa JP, George JN, Bainton DF, Nurden AT, Caen JP, McEver RP. Gray platelet syndrome: demonstration of alpha-granule membranes that can fuse with the cell surface. *J Clin Invest* 1987; 80: 1138–1146.
113 Pfueller SL, David R. Platelet-associated immunoglobulin G, A and M are secreted during platelet activation: normal levels but deficient secretion in grey platelet syndrome. *Br J Haematol* 1988; 68: 235–241.
114 Cramer EM, Savidge GF, Vainchenker G et al. Alpha granule pool of glycoprotein IIb–IIIa in normal and pathologic platelets and megakaryocytes. *Blood* 1990; 75: 1220–1227.
115 Berger G, Caenm JP, Berndt MC, Cramer EM. Ultrastructural demonstration of CD36 in the anule membrane of human platelets and megakaryocytes. *Blood* 1993; 82: 3034–3044.
116 Berger G, Massé JM, Cramer EM. Alpha-granule membrane mirrors the platelet plasma membrane and contains the glycoproteins Ib, IX, and V. *Blood* 1996; 87: 1385–1395.
117 Cramer EM, Vainchenker G, Vinci J, Guichard J, Breton-Gorius J. Gray platelet syndrome: immunoelectron microscopic localization of fibrinogen and vWf in platelets and megokaryocytes. *Blood* 1985; 66: 1309–1316.
118 Breton-Gorius J, Vainshenker W, Nurden AT, Levy-Toledano S, Caen JP. Defective alpha-granule production in megakaryocytes from gray platelet syndrome: ultrastructural studies of bone marrow cells and megakaryocytes growing in culture from blood precursors. *Am J Pathol* 1981; 102: 10–19.
119 Levy-Toledano S, Caen JP, Breton-Gorius J et al. Gray platelet syndrome: alpha-granule deficiency. Its influence on platelet function. *J Lab Clin Med* 1981; 98: 831–848.
120 Gebrane-Younès J, Cramer EM, Orcel L, Caenm JP. Gray platelet syndrome. Dissociation between abnormal sorting in megakaryocyte α-granules and normal sorting in Weibel–Palade bodies of endothelial cells. *J Clin Invest* 1993; 92: 3023–3028.
121 White JG. Ultrastructural studies of the gray platelet syndrome. *Am J Pathol* 1979; 95: 445–462.
122 Srivastava PC, Powling MJ, Nokes TJC, Patrick AD, Dawes J, Hardisty RM. Gray platelet syndrome: studies on alpha granules, lysosomes and defective response to thrombin. *Br J Haematol* 1987; 65: 441–446.
123 Lages B, Sussman II, Levine SP, Coletti D, Weiss HJ. Platelet alpha granule deficiency associated with decreased P-selectin and selective impairment of thrombin-induced activation in a new patient with grey platelet syndrome (α-storage pool deficiency). *J Lab Clin Med* 1997; 129: 364–375.
124 Alberio L, Safa O, Clemetson KJ, Esmon CT, Dale GL. Surface expression and functional characterization of alpha-granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin. *Blood* 2000; 95: 1694–1702.
125 Jackson CW, Hutsonm NK, Steward SA, Saito N, Cramer EM. Platelets of the Wistar Furth rat have reduced levels of alpha-granule proteins. An animal model resembling gray platelet syndrome. *J Clin Invest* 1991; 87: 1985–1991.
126 Stenberg PE, Barrie RJ, Pestina TI, Steward SA, Arnold JT, Murti AK, Hutson NK, Jackson CW. Prolonged bleeding time with defective platelet filopodia formation in the Wistar Furth rat. *Blood* 1998; 91: 1599–1608.
127 Stenberg PE, McDonald TP, Jackson CW. Disruption of microtubules in vivo by vincristine induces large membrane complexes and other cytoplasmic abnormalities in megakaryocytes and platelets of normal rats like those in human and Wistar Furth rat hereditary macrothrombocytopenias. *J Cell Physiol* 1995; 162: 86–102.
128 Tracy PB, Giles AR, Mann KG, Eide LL, Hoogendoorn H, Rivard GE. Factor V (Quebec): a bleeding diathesis associated with a qualitative platelet factor V deficiency. *J Clin Invest* 1984; 74: 1221–1228.
129 Hayward CP, Rivard GE, Kane WH. An autosomal dominant, qualitative platelet disorder associated with multimerin deficiency, abnormalities in platelet factor V, thrombospondin,

Cambridge Books Online © Cambridge University Press, 2009

von Willebrand factor, and fibrinogen, and an epinephrine aggregation defect. *Blood* 1996; 87: 4967–4978.

130 Hayward CPM, Warkentin TE, Horsewood P, Kelton JG. Multimerin: a series of large, disulfide-linked multimeric proteins within platelets. *Blood* 1991; 77: 2556–2560.

131 Hayward CPM, Bainton DF, Smith JW et al. Multimerin is found in the α-granules of resting platelets and is synthesized by a megakaryocytic cell line. *J Clin Invest* 1993; 91: 2630–2639.

132 Hayward CPM, Hassell JA, Denomme GA, Rachubinski RA, Brown C, Kelton JG. The cDNA sequence of human endothelial cell multimerin: A unique protein with RGDS, coiled-coil, and EGF-like domains and a carboxyl-terminus similar to the globular domain of complement C1q and collagens type VIII and X. *J Biol Chem* 1995; 270: 19217–19224.

133 Janeway CM, Rivard GE, Tracy PB, Mann KG. Factor V Quebec revisited. *Blood* 1996; 87: 3571–3578.

134 Hayward CPM, Cramer EM, Kane WH et al. Studies of a second family with the Quebec Platelet Disorder: evidence that the degradation of the α-granule membrane and its soluble contents are not secondary to a defect in targeting proteins to α-granules. *Blood* 1997; 89: 1243–1253.

135 Breton-Gorius J, Favier R, Guichard J et al. A new congenital dysmegakaryopoietic thrombocytopenia (Paris-Trousseau) associated with giant platelet alpha-granules and chromosome 11 deletion at 11q23. *Blood* 1995; 85: 1805–1814.

136 Hart A, Melet F, Grossfeld P et al. *Fli*-1 is required for murine vascular and megakaryocytic development and is hemizygously deleted in patients with thrombocytopenia. *Immunity* 2000; 13: 167–177.

137 Cockbill SR, Burmester HB, Heptinstall S. Pseudo grey platelet syndrome – grey platelets due to degranulation in blood collected into EDTA. *Eur J Haematol* 1988; 41: 326–333.

138 Lages B, Shattil SJ, Bainton DF, Weiss HJ. Decreased content and surface expression of alpha-granule membrane protein GMP-140 in one of two types of platelet alpha delta storage pool deficiency. *J Clin Invest* 1991; 87: 919–920.

139 Vicic WJ, Weiss HJ. Evidence that platelet alpha-granules are a major determinant of platelet density: studies in storage pool deficiency. *Thromb Haemost* 1983; 50: 878–880.

140 Swank RT, Jiang SY, Reddington M et al. Inherited abnormalities in platelet organelles and platelet formation and associated altered expression of low molecular weight guanosine triphosphate-binding proteins in the mouse pigment mutant gunmetal. *Blood* 1993; 81: 2626–2635.

141 Novak EK, Reddington M, Zhen L et al. Inherited thrombocytopenia caused by reduced platelet production in mice with the gunmetal pigment gene mutation. *Blood* 1995; 85: 1781–1789.

142 Holmsen H, Walsh PN, Koike K et al. Familial bleeding disorder associated with deficiencies in platelet signal processing and glycoproteins. *Br J Haematol* 1987; 67: 335–344.

143 Rao AK, Koike K, Willis J et al. Platelet secretion defect associated with impaired liberation of arachidonic acid and normal myosin light chain phosphorylation. *Blood* 1984; 64: 914–921.

144 Rao AK, Disa, J, Yang X. Concomitant defect in internal release and influx of calcium in patients with congenital platelet dysfunction and impaired agonist-induced calcium mobilization. Thromboxane production is not required for internal release of calcium. *J Lab Clin Med* 1993; 121: 52–63.

145 Gabbeta J, Yang X, Kowalska MA, Sun L, Dhanasekaran N, Rao AK. Platelet signal transduction defect with Gα subunit dysfunction and diminished Gαq in a patient with abnormal platelet responses. *Proc Natl Acad Sci USA* 1997; 94: 8750–8755.

146 Roth GJ, Machuga ET. Radioimmuno assay of human platelet prostaglandin synthetase. *J Lab Clin Med* 1982; 99: 187–196.

147 Fuse I, Hattori A, Nagayama R. Characterization of platelet cytoplasmic Ca^{2+} mobilization in patients with congenital cyclo-oxygenase deficiency and with defective platelet aggregation to A23187. *Nippon Ketsueki Gakkai Zasshi.* 1989; 52: 1522–1533.

148 Pareti FI, Mannucci PM, D'Angelo A, Smith JB, Sautebin L, Galli G. Congenital deficiency of thromboxane and prostacyclin. *Lancet* 1980; 1(8174): 898–901.

149 Dechavanne M, Lagarde M, Follea G, Ville D. Post-surgical deep vein thrombosis in woman with congenital platelet cyclo-oxygenase deficiency. *Br J Haematol* 1978; 40: 168.

150 Lagarde M, Byron PA, Vargaftig BB, Dechavanne M. Impairment of platelet thromboxane A2 generation and of the platelet release reaction in two patients with congenital deficiency of platelet cyclo-oxygenase. *Br J Haematol* 1978; 38: 251–266.

151 Nakanishi K, Ikeda K, Hato T et al. Platelet cyclo-oxygenase deficiency in a Japanese. *Scand J Haematol* 1984; 32: 167–174.

152 Horellou MH, Lecompte T, Lecrubier C et al. Familial and constitutional bleeding disorder due to platelet cyclo-oxygenase deficiency. *Am J Haematol* 1983; 14: 1–9.

153 Malmsten C, Hamberg M, Svensson J, Samuelsson B. Physiological role of an endoperoxide in human platelets: hemostatic defect due to platelet cyclo-oxygenase deficiency. *Proc Natl Acad Sci USA* 1975; 72: 1446–1450.

154 Rak K, Boda Z. Haemostatic balance in congenital deficiency of platelet cyclo-oxygenase. *Lancet* 1980; 2: 44.

155 Flick JT, Singh AK, Kizer J, Lazarchick J. Platelet dysfunction in Noonan's syndrome. A case with a platelet cyclooxygenase-like deficiency and chronic idiopathic thrombocytopenic purpura. *Am J Clin Pathol* 1991; 95: 739–742.

156 Roth GJ, Majerus PW. The mechanism of the effect of aspirin on human platelets. I. Acetylation of a particulate fraction protein. *J Clin Invest* 1975; 56: 624–632.

157 Roth GJ, Stanford N, Majerus PW. Acetylation of prostaglandin synthase by aspirin. *Proc Natl Acad Sci USA* 1975; 72: 3073–3076.

158 Matijevic-Aleksic N, McPhedran P, Wu KK. Bleeding disorder due to platelet prostaglandin H synthase-1 (PGHS-1) deficiency. *Br J Haematol* 1996; 92: 212–217.

159 Marcus AJ. Aspirin and thromboembolism – a possible dilemma. *N Engl J Med* 1977; 297: 1284–1285.

160 Defreyn G, Machin SJ, Carreras LO, Dauden MV, Chamone

Cambridge Books Online © Cambridge University Press, 2009

DA, Vermylen J. Familial bleeding tendency with partial platelet thromboxane synthetase deficiency: reorientation of cyclic endoperoxide metabolism. *Br J Haematol* 1981; 49: 29–41.

161 Mestel F, Oetliker O, Beck E, Felix R, Imbach P, Wagner HP. Severe bleeding associated with defective thromboxane synthetase. *Lancet* 1980; 1: 157.

162 Lages B, Malmsten C, Weiss HJ, Samuelsson B. Impaired platelet response to thromboxane-A2 and defective calcium mobilization in a patient with a bleeding disorder. *Blood.* 1981; 57: 545–552.

163 Samama M, Lecrubier C, Conard J et al. Constitutional thrombocytopathy with subnormal response to thromboxane A2. *Br J Haematol* 1981; 48: 293–303.

164 Wu KK, Le Breton GC, Tai HH, Chen YC. Abnormal platelet response to thromboxane A2. *J Clin Invest* 1981; 67: 1801–1804.

165 Fuse I, Mito M, Hattori A et al. Defective signal transduction induced by thromboxane A2 in a patient with a mild bleeding disorder: impaired phospholipase C activation despite normal phospholipase A2 activation. *Blood* 1993; 81: 994–1000.

166 Ushikubi F, Okuma M, Kanaji K et al. Hemorrhagic thrombocytopathy with platelet thromboxane A2 receptor abnormality: defective signal transduction with normal binding activity. *Thromb Haemost* 1987; 57: 158–164.

167 Hirata T, Kakizuka A, Ushikubi F, Fuse I, Okuma M, Narumiya S. Arg60 to Leu mutation of the human thromboxane A2 receptor in a dominantly inherited bleeding disorder. *J Clin Invest* 1994; 94: 1662–1667.

168 Hirata T, Ushikubi F, Kakizuka A, Okuma M, Narumiya S. Two thromboxane A2 receptor isoforms in human platelets. Opposite coupling to adenylyl cyclase with different sensitivity to Arg60 to Leu mutation. *J Clin Invest* 1996; 97: 949–956.

169 Okuma M, Hirata T, Ushikubi F, Kakizuka A, Narumiya S. Molecular characterization of a dominantly inherited bleeding disorder with impaired platelet responses to thromboxane A2. *Pol J Pharmacol* 1996; 48: 77–82.

170 Fuse I, Hattori A, Mito M, Higuchi W, Yahata K, Shibata A, Aizawa Y. Pathogenetic analysis of five cases with a platelet disorder characterized by the absence of thromboxane A2 (TXA2)-induced platelet aggregation in spite of normal TXA2 binding activity. *Thromb Haemost* 1996; 76: 1080–1085.

171 Higuchi W, Fuse I, Hattori A, Aizawa Y. Mutations of the platelet thromboxane A2 (TXA2) receptor in patients characterized by the absence of TXA2-induced platelet aggregation despite normal TXA2 binding activity. *Thromb Haemost* 1999; 82: 1528–1531.

172 Chignard M, Vargaftig BB. Synthesis of thromboxane A2 by non-aggregating dog platelets challenged with arachidonic acid or with prostaglandin H2. *Prostaglandins* 1977; 14: 222–240.

173 Johnson GJ, Leis LA, Dunlop PC. Thromboxane-insensitive dog platelets have impaired activation of phospholipase C due to receptor-linked G protein dysfunction. *J Clin Invest* 1993; 92: 2469–2479.

174 Johnson GJ, Leis LA, Rao GHR, White JG. Arachidonate-induced platelet aggregation in the dog. *Thromb Res* 1979; 14: 147–154.

175 Johnson GJ, Rao GHR, Leis LA, White JG. Effect of agents that alter cyclic AMP on arachidonate-induced platelet aggregation in the dog. *Blood* 1980; 55: 722–729.

176 Dunlop PC, Leis LA, Johnson GJ. Epinephrine correction of impaired platelet thromboxane receptors signaling. *Am J Physiol Cell Physiol* 2000; 279: C1760–C1771.

177 Thomas DW, Mannon RB, Mannon PJ et al. Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2. *J Clin Invest* 1998; 102: 1994–2001.

178 Daniel JL, Dangelmaier CA, Jin J, Ashby B, Smith JB, Kunapuli SP. Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. *J Biol Chem* 1998; 273: 2024–2029.

179 Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. *Proc Natl Acad Sci USA* 1998; 95: 8070–8074.

180 Geiger J, Honig-Liedl, Schanzenbacher P, Walter U. Ligand specificity and ticlopidine effects distinguish three human platelet ADP receptors. *Eur J Pharmacol* 1998; 351: 235–246.

181 Hechler B, Léon C, Vial C et al. The $P2Y_1$ receptor is necessary for ADP-induced platelet aggregation. *Blood* 1998; 92: 152–159.

182 Jantzen H-M, Gousset L, Bhaskar V, Vincent D, Tai A, Reynolds EE, Conley PB. Evidence for two distinct G-protein-coupled ADP receptors mediating platelet activation. *Thromb Haemost* 1999; 81: 111–117.

183 Cattaneo M, Gachet C. ADP receptors and clinical bleeding disorders. *Arterioscler Thromb Vasc Biol* 1999; 19: 2281–2285.

184 Hollopeter G, Jantzen H-M, Vincent D et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* 2001; 409: 202–207.

185 Zhang FL, Luo L, Gustafson E et al. ADP is the cognate ligand for the orphan G-protein coupled receptor SP1999. *J Biol Chem* 2001; 276: 8608–8615.

186 Oury C, Toth-Zsamboki E, Van Geet C et al. A natural dominant negative P2X1 receptor due to deletion of a single amino acid residue. *J Biol Chem* 2000; 275: 22611–22614.

187 Mahaut-Smith MP, Ennion SJ, Rolf MG, Evans RJ. ADP is not an agonist at P2X1 receptors: evidence for separate receptors stimulated by ATP and ADP on human platelets. *Br J Pharmacol* 2000; 131: 108–114.

188 Cattaneo M, Lecchi A, Randi AM, McGregor JL, Mannucci PM. Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. *Blood* 1992; 80: 2787–2796.

189 Gachet C, Cattaneo M, Ohlmann P et al. Purinoceptors on blood platelets: further pharmacological and clinical evidence to suggest the presence of two ADP receptors. *Br J Haematol* 1995; 91: 434–444.

190 Nurden P, Savi P, Heilmann E et al. An inherited bleeding disorder linked to a defective interaction between ADP and its receptor on platelets. *J Clin Invest* 1995; 95: 1612–1622.

Cambridge Books Online © Cambridge University Press, 2009

191 Levy-Toledano S, Maclouf J, Rosa JP et al. Abnormal tyrosine phosphorylation linked to a defective interaction between ADP and its receptos on platelets. *Thromb Haemost* 1998; 80: 463–468.
192 Cattaneo M. Hereditary defect of the platelet ADP receptor(s). *Platelets* 1998; 8: 161–164.
193 Weiss HJ. Congenital disorders of platelet function. *Sem Haematol* 1980; 17: 228–241.
194 Rao AK, Gabbeta J. Congenital disorders of signal transduction. *Arterioscler Thromb Vasc Biol* 2000; 20: 285–289.
195 Rao AK. Congenital disorders of platelet secretion and signal transduction. In Colman RW, Hirsh J, Marder VJ, Clowes AW, George JN, eds. *Hemostasis and Thrombosis. Basic Principles and Clinical Practice.* Philadelphia, PA: J.B. Lippincott Williams and Wilkins; 2001: 893–904.
196 Wu KK, Minkoff IM, Rossi EC, Chen YC. Hereditary disorder due to a primary defect in platelet release reaction. *Br J Haematol* 1981; 47: 241–249.
197 Hardisty RM, Machin SJ, Nokes TJ, Rink TJ, Smith SW. A new congenital defect of platelet secretion: impaired responsiveness of the platelets to cytoplasmic free calcium. *Br J Haematol* 1983; 53: 543–557.
198 Moss HB, Yao JK, Lynch K. Platelet dense granule secretion and aggregation in adolescents with conduct disorder: effects of marijuana use. *Biol Psychiatry* 1999; 46: 790–798.
199 Koike K, Rao K, Holmsen H, Mueller PS. Platelet secretion defect in patients with the attention deficit disorder and easy bruising. *Blood* 1984; 63: 427–433.
200 Rao AK, Kowalska MA, Disa J. Impaired calcium mobilization in inherited platelet secretion defects. *Blood* 1989; 74: 664–672.
201 Yang X, Sun L, Ghosh S, Rao AK. Human platelet signaling defect characterized by impaired production of 1,4,5 inositol triphosphate and phosphatidic acid, and diminished pleckstrin phosphorylation. Evidence for defective phospholipase C activation. *Blood* 1996; 88: 1676– 1683.
202 Lee SB, Rao AK, Lee K-H, Yang X, Bae YS, Rhee SG. Decreased expression of phospholipase C-β2 isozyme in human platelets with impaired function. *Blood* 1996; 88: 1676–1691.
203 Lages B, Weiss HJ. Heterogeneous defects of platelet secretion and responses to weak agonists in patients with bleeding disorders. *Br J Haematol* 1988; 68: 53–62.
204 Wijermans PW, van Dorp DB. Hermansky–Pudlak syndrome: correction of bleeding time by 1-desamino-8D-arginine vasopressin. *Am J Haematol* 1989; 30: 154–157.
205 Cattaneo M, Mannucci PM. Current status of non-transfusional haemostatic agents. *Haematologica* 1999; 84(Supplt): 120–123.
206 Kobrinsky NL, Israels ED, Gerrard JM et al. Shortening of bleeding time by 1-deamino-8–D-arginine vasopressin in various bleeding disorders. *Lancet* 1984; 1: 1145–1148.
207 Mannucci PM, Vicente V, Vianello L et al. Controlled trial of desmopressin in liver cirrhosis and other conditions associated with a prolonged bleeding time. *Blood* 1986; 67: 1148–1153.
208 Pfueller SL, Howard MA, White JG, Menon C, Berry EW. Shortening of bleeding time by 1-deamino-8-arginine vasopressin (DDAVP) in the absence of platelet von Willebrand factor in Gray platelet syndrome. *Thromb Haemost* 1987; 58: 1060–1063.
209 Nieuwenhuis HK, Sixma JJ. 1–Desamino-8–D-arginine vasopressin (desmopressin) shortens the bleeding time in storage pool deficiency. *Ann Intern Med* 1988; 108: 65–67.
210 Rao AK, Ghosh S, Sun L et al. Mechanisms of platelet dysfunction and response to DDAVP in patients with congenital platelet function defects. A double-blind placebo-controlled trial. *Thromb Haemost* 1995; 74: 1071–1078.
211 Mannucci PM. Drug therapy: hemostatic drugs. *N Engl J Med* 1998; 339: 245–253.
212 Cattaneo M, Zighetti ML, Lombardi R, Mannucci PM. Role of ADP in platelet aggregation at high shear: studies in a patient with congenital defect of platelet responses to ADP. *Br J Haematol* 1994; 88: 826–829.
213 Cattaneo M, Moia M, Della Valle P, Castellana P, Mannucci PM. DDAVP shortens the prolonged bleeding times of patients wwith severe von Willebrand Disease treated with cryoprecipitate. Evidence for a mechanism of action independent of released von Willebrand factor. *Blood* 1989; 74: 1972–1976.
214 Rolf MG, Brearley CA, Mahaut-Smith MP. Platelet shape change evoked by selective activation of P2X1 purinoceptors with α,β-methylene ATP. *Thromb Haemost* 2001; 85: 303–308.

Cambridge Books Online © Cambridge University Press, 2009

44

Congenital platelet signal transduction defects

A. Koneti Rao

Departments of Medicine, Sol Sherry Thrombosis Research Center, and Pathology
Temple University School of Medicine, Philadelphia, PA 19140 USA

Platelets play a major role in hemostasis, and alterations in platelet mechanisms may lead to a bleeding diathesis. When the blood vessel is injured, platelets adhere to exposed subendothelium by a process (adhesion) which involves the interaction of a plasma protein, von Willebrand factor (vWF), and a specific protein on the platelet surface, glycoprotein Ib (GPIb) (Fig. 44.1). Adhesion is followed by recruitment of additional platelets which form clumps, a process called aggregation (cohesion). This involves binding of fibrinogen to specific platelet surface receptors, a complex composed of glycoproteins IIb–IIIa (GPIIb–IIIa). Activated platelets release contents of their granules (secretion or release reaction), such as adenosine diphosphate (ADP) and serotonin from the dense granules, which cause recruitment of additional platelets. In addition, platelets play a major role in coagulation mechanisms; several key enzymatic reactions occur on the platelet membrane lipoprotein surface. A number of physiological agonists interact with specific receptors on platelet surface to induce responses including a change in platelet shape from discoid to spherical (shape change), aggregation, secretion, and thromboxane A_2 (TxA_2) production. Other agonists, such as prostacyclin, inhibit these responses. Ligation of the platelet receptors initiates the production or release of several intracellular messenger molecules including Ca^{2+} ions, products of phosphoinositide (PI) hydrolysis by phospholipase C (diacylglycerol, DG, and inositol 1,4,5-triphosphate, $InsP_3$), TxA_2 and cyclic nucleotides (cAMP) (Fig. 44.1). These induce or modulate the various platelet responses of Ca^{2+} mobilization, protein phosphorylation, aggregation, secretion and liberation of arachidonic acid. The interaction between the agonist receptors and the key intracellular effector enzymes (e.g. phospholipases A_2 and C, adenylyl cyclase) is mediated by a group of GTP-binding proteins which are modulated by GTP. As in most secretory cells, platelet activation results in a rise in cytoplasmic ionized calcium concentration; $InsP_3$ functions as a messenger to mobilize Ca^{2+} from intracellular stores. Diacylglycerol activates protein kinase C (PKC) and this results in the phosphorylation of a 47 kD protein pleckstrin. PKC activation is considered to play a major role in platelet secretion and in the activation of GP IIb–IIIa. Numerous other mechanisms, such as phosphorylation of proteins by non-receptor tyrosine kinases, also play a role in signal transduction. A detailed description of the platelet activation mechanisms is provided elsewhere in this book. Inherited defects in the above platelet mechanisms may lead to impaired platelet role in hemostasis and are described in this chapter.

Congenital disorders of platelet function

In general, congenital disorders of platelet function are characterized by highly variable mucocutaneous bleeding manifestations and excessive hemorrhage following surgical procedures or trauma. A majority of patients, but not all, have a prolonged bleeding time. Platelet aggregation and secretion studies provide evidence for the defect, but are not always predictive of the severity of clinical manifestations. The platelet dysfunction in these patients arises by diverse mechanisms[1,2]. Table 44.1 provides a classification based on the platelet functions or responses that are abnormal (Fig. 44.1). In patients with defects in platelet–vessel wall interactions, adhesion of platelets to subendothelium is abnormal. The two disorders in this group are the von Willebrand disease (vWD), due to a deficiency or abnormality in plasma vWF[3], and the Bernard–Soulier syndrome, where platelets are deficient in GPIb (and GPV and

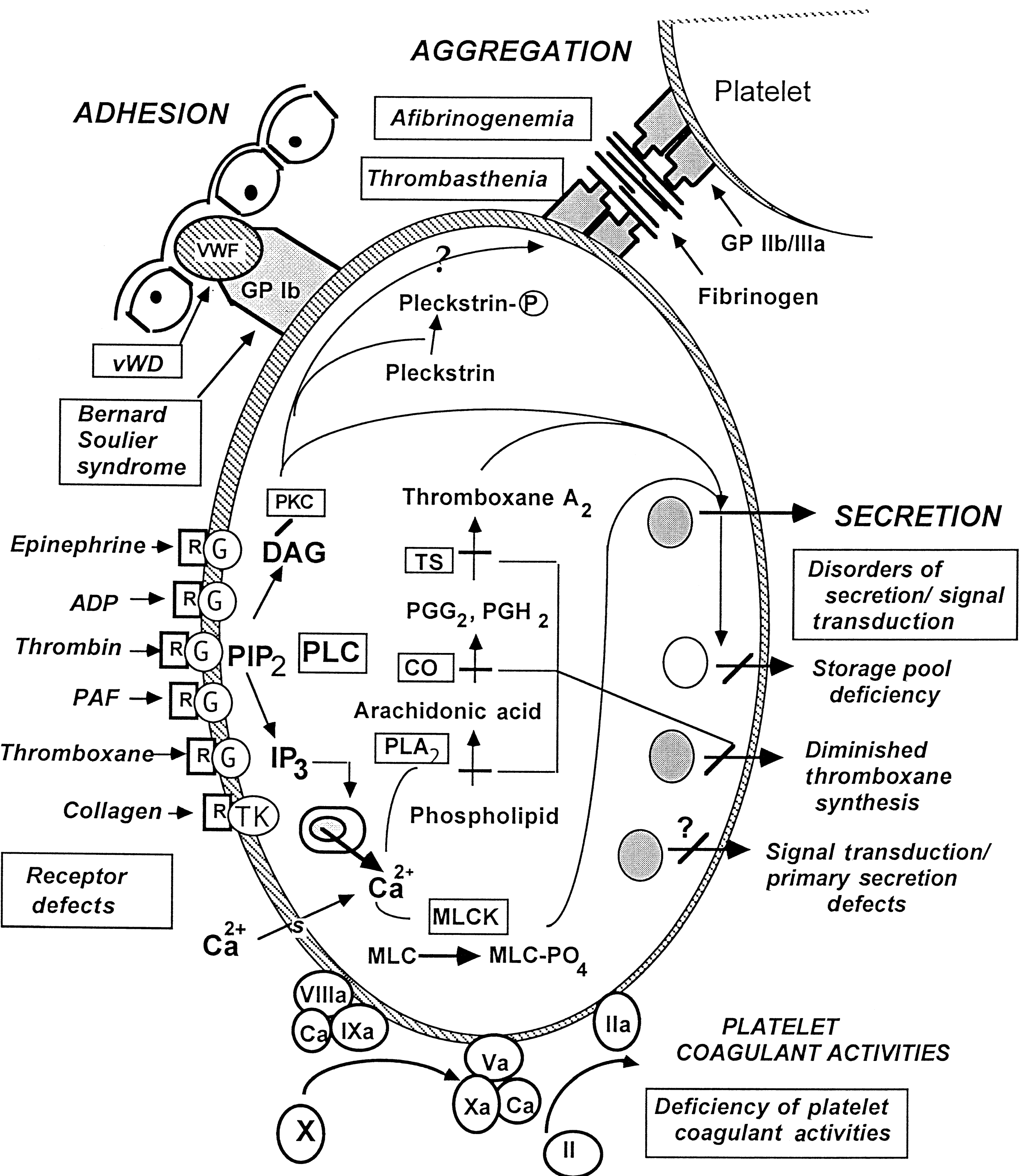


Fig. 44.1. A schematic representation of the normal platelet responses and the congenital disorders of platelet function. Abbreviations: CO, cyclooxygenase; DAG, diacylglycerol; IP_3, inositoltrisphosphate; MLC, myosin light chain; MLCK, myosin light chain kinase; PIP_2, phosphatidylinositol bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PLA_2, phospholipase A_2; TS, thromboxane synthase; vWF, von Willebrand factor; vWD, von Willebrand disease. (Reprinted with permission[1].)

Cambridge Books Online © Cambridge University Press, 2009

Table 44.1. Classification of congenital disorders of platelet function

1. Defects in platelet-vessel wall interaction (disorders of adhesion)
   (*a*) von Willebrand disease (deficiency or defect in plasma vWF)
   (*b*) Bernard–Soulier syndrome (deficiency or defect in GPIb)
2. Defects in platelet-platelet interaction (disorders of aggregation)
   (*a*) Congenital afibrinogenemia (deficiency of plasma fibrinogen)
   (*b*) Glanzmann thrombasthenia (deficiency or defect in GPIIb–IIIa)
3. Disorders of platelet secretion and signal transduction
   (*a*) Abnormalities of granules
      (i) Storage pool deficiency
      (ii) Quebec platelet disorder
   (*b*) Signal transduction defects (primary secretion defects)
      (i) Defects in platelet–agonist interaction (receptor defects)
      Receptor defects: thromboxane A_2, collagen, ADP, epinephrine
      (ii) Defects in G-protein activation
      Gαq deficiency
      (iii) Defects in phosphatidylinositol metabolism
      Phospholipase C-β2 deficiency
      (iv) Defects in calcium mobilization
      (v) Defects in protein phosphorylation (pleckstrin)
   (*c*) Abnormalities in arachidonic acid pathways and thromboxane A_2 synthesis
      (i) Impaired liberation of arachidonic acid
      (ii) Cyclooxygenase deficiency
      (iii) Thromboxane synthase deficiency
   (*d*) Defects in cytoskeletal regulation
   Wiskott–Aldrich syndrome
4. Disorders of platelet coagulant-protein interaction
   Defect in factor Va–Xa interaction on platelets (Scott syndrome)

Source: Reprinted with permission from ref [1].

IX) and the binding of vWF to platelets is abnormal[4]. Disorders characterized by abnormal platelet–platelet interactions (aggregation) arise because of an absence of plasma fibrinogen (congenital afibrinogenemia) or because of a quantitative or qualitative abnormality of the platelet membrane GPIIb–IIIa complex (Glanzmann thrombasthenia)[5]. Patients with defects in platelet secretion and signal transduction are a heterogeneous group lumped together for convenience of classification rather than on the basis of an understanding of the specific underlying abnormality. The major common characteristic in these patients, as currently perceived, is an inability to release intracellular granule (dense) contents upon activation of platelet-rich plasma with agonists such as ADP, epinephrine and collagen. In aggregation studies the second wave of aggregation is blunted or absent. A small proportion of these patients have a deficiency of dense granule stores (storage pool deficiency). In some of the other patients, the impaired secretion results from aberrations in the signal transduction events that govern end responses such as secretion and aggregation. This review will focus on these patients who are encountered more often than thrombasthenia or the Bernard–Soulier syndrome. Lastly, are the patients who have an abnormality in interactions of platelets with proteins of the coagulation system; the best described is the Scott syndrome[6]. In addition to the above groups, there are patients who have abnormal platelet function associated with systemic disorders such as Down syndrome and the May–Hegglin anomaly where the specific aberrant platelet mechanisms are still unclear.

Disorders of platelet secretion and signal transduction

As an unifying theme, patients lumped in this remarkably heterogeneous group generally manifest impaired secretion of granule contents and absence of the second wave of aggregation upon stimulation of platelet-rich plasma with ADP and epinephrine; responses to collagen, thromboxane analogue (U46619), arachidonic acid, and platelet-activating factor (PAF) may also be impaired. Platelet function is abnormal in these patients either when the granule contents are diminished (storage pool deficiency, SPD) or when there is an aberration in the activation mechanisms governing aggregation and secretion, the easily discernible end-responses following platelet activation by a diverse group of agonists. (Table 44.1).

Deficiency of granule stores: storage pool deficiency

Patients with storage pool deficiency (SPD) have deficiencies in platelet content of dense granules (δ-SPD), alpha-granules (α-SPD) or both types of granules ($\alpha\delta$-SPD)[7,8]. The Quebec platelet disorder is an autosomal dominant disorder associated with abnormal proteolysis of α-granule proteins, deficiency of platelet α-granule multimerin (a factor V binding protein), and markedly

Cambridge Books Online © Cambridge University Press, 2009

impaired aggregation with epinephrine as a striking feature[8]. These disorders are discussed elsewhere in detail.

Defects in platelet signal transduction (primary secretion defects)

Signal transduction mechanisms encompass processes that are initiated by the interaction of agonists with specific platelet receptors and include responses such as G-protein activation and activation of effectors such as phospholipase C and phospholipase A_2. If the key components in signal transduction are the surface receptors, the G-proteins, and the effectors, evidence now exists for specific platelet abnormalities at each of these levels. It is only a matter of time before deficiencies are documented in the various specific mechanisms involving other proteins, for example, the kinases and phosphatases, that are activated on platelet receptor ligation.

Defects in platelet–agonist interaction: receptor defects

These patients are characterized by impaired platelet responses resulting from an abnormality at the level of platelet surface receptors for a specific agonist. Such receptor defects have been documented for TxA_2, collagen, ADP and epinephrine. One patient has been described with diminished responses to PAF alone[9]. Because ADP and TxA_2 play a synergistic role in the platelet responses to several agonists, it is not surprising that patients with specific defects at the ADP or TxA_2 receptor have impaired responses to multiple agonists, particularly the weaker ones.

Thromboxane A2 receptor defect

In some patients with platelet dysfunction[10–12] the abnormal aggregation responses have been attributed to an impaired platelet responsiveness to TxA_2, although it is synthesized in normal amounts. Because TxA_2 is highly unstable, these studies have used a system in which TxA_2 was generated by incubating normal platelets with arachidonic acid and aliquots of the stimulated platelet suspension or its supernate were transferred to the patient's platelets as a source of TxA_2. Based on a diminished response to stimulation with such aliquots, the platelet dysfunction has been attributed to an impaired platelet responsiveness to TxA_2 or to a TxA_2 receptor defect[10–12]. However, in such a test system, the responses induced in the recipient platelets occur due to a synergism between TxA_2 and ADP and not due to the former alone, and they are dependent on the ability of the test platelets to secrete ADP[13]. Therefore, the primary defect in these patients[10,11] is not established to be a specific impairment in the response to TxA_2. In one of these patients[12], TxA_2 analogue U46619 failed to suppress PGI_2 induced cAMP levels, suggesting that this patient may have a defect at the TxA_2 receptor level. In another patient[10], subsequent studies[14] have revealed abnormalities in the phosphatidylinositol metabolism on platelet activation with thrombin and collagen.

The existence of specific mutations in the platelet TxA_2 receptor has been established by Hirata et al.[15] who described an Arg^{60} to Leu mutation in the first cytoplasmic loop of the TxA_2 receptor in two unrelated patients. This Arg^{60} corresponds to a highly conserved basic residue among G-protein coupled receptors[15]. These patients had a mild bleeding disorder with an autosomal dominant pattern of inheritance. Aggregation responses to several agonists were impaired with the exception of thrombin[16]. The binding of TxA_2 analogues to platelets was normal[16,17]. GTPase activity on activation with a TxA_2 analog, but not thrombin, was diminished[17,18] suggesting a defect in TxA_2 receptor-G-protein coupling. TxA_2-induced activation of PLC (measured as Ca^{2+} mobilization, inositol trisphosphate, and phosphatidic acid formation) was impaired while PLA_2 activation and TxA_2 production were normal. Less than half the number of TxA_2 receptors are sufficient for irreversible aggregation with TxA_2 agonist[19]. The finding that the aggregation responses were impaired in the heterozygous family members[15] suggest a dominant negative effect of the mutation. It needs to be noted that absent aggregation responses to TxA_2 have been observed in patients who do not have evidence for a defect of TxA_2 receptor level[20]. Lastly, thromboxane receptor knockout mice have a mild bleeding disorder and their platelets have impaired aggregation responses to TxA2 analogues as well as collagen[21].

Collagen receptor defects

Several patients have been reported with mucocutaneous bleeding manifestations and selective impairment in platelet-collagen interaction. Platelets from the patient described by Nieuwenhuis et al.[22,23] had ~ 15 to 25% of normal amount of platelet GPIa and failed to aggregate with collagen, or adhere and spread normally to subendothelial surfaces. In another patient described by Kehrel et al.[24], collagen-induced platelet aggregation was markedly reduced, and the platelets were deficient in GPIa and thrombospondin. In both of these patients, the bleeding times were prolonged and platelet aggregation responses to other agonists were preserved. Selective impairment in collagen responses and a mild bleeding disorder have also

Cambridge Books Online © Cambridge University Press, 2009

been related to a deficiency of platelet GP VI[25–27]. GP VI deficient platelets have been reported to have impaired collagen activation of Syk but not c-Src[28]. In the current models of platelet–collagen interactions, collagen binds initially to either the GPIa–GPIIa ($\alpha 2\beta 1$) or GPVI, leading to subsequent binding to the other receptor which serves to reinforce adhesion and generation of intracellular signals[29].

GP IV (CD 36) has also been implicated in platelet–collagen interactions[30]. Platelets lacking GP IV have been reported to have reduced adhesion to collagen in flowing whole blood[31]. However, individuals lacking platelet GP IV in the Japanese population (approximately 3% of the population) and the US population (approximately 0.3%) have not had a bleeding disorder[32] and collagen-induced platelet aggregation[32], Ca^{2+} mobilization, and tyrosine phosphorylation[33] have been normal. In other studies[34] GP IV deficient platelets aggregated normally in response to collagens type I and III but not to type V collagen. Moreover, adhesion and subsequent aggregate formation of GP IV deficient platelets on types I, III and IV collagens were normal under static or flow conditions whereas adhesion to type V collagen was reduced under static conditions[35]. In several GP IV deficient subjects a Pro^{90} to ser mutation has been reported in the GP IV gene[36] and GP IV mRNA has been detected in platelets from all subjects studied[36,37]. Kehrel et al.[34] have described platelet responses to collagen-related peptides; platelets deficient in GP VI but not GP IV failed to aggregate in response to these peptides.

Defect in platelet ADP receptors

ADP interaction with platelets is mediated by multiple receptors (P2Y1, $P2T_{AC}/P2Y_{12}$, P2X1) which elicit distinct responses[38–40]. Cattaneo et al.[41] and Nurden et al.[42] have described two patients in whom platelet aggregation response to ADP was blunted (but not absent) and the ability of ADP to suppress PGE_1-induced elevation in cAMP levels was impaired; ADP stimulated shape change was normal. ADP stimulated Ca^{2+} mobilization and tyrosine phosphorylation in response to ADP and TxA_2 have been reported to be abnormal in the patients studied[41,43]. Aggregation in response to a TxA_2 analogue and lower concentrations of collagen were also impaired. The binding of radiolabelled ADP[41] or the ADP analogue 2-methylthio-ADP[42] to platelets was decreased in these patients. Recent studies[44] in one of these patients,[42] provide evidence of a mutation in the $P2T_{AC}$ ($P2Y_{12}$) ADP receptor linked to inhibition of adenyl cyclase. Electron microscopic studies in one patient with the ADP receptor defect[45] showed that the aggregates formed following stimulation with high dose ADP were composed of loosely bound platelets with few contact points. A decreased platelet 2-methylthio-ADP binding has been reported[46–48] in additional patients with impaired aggregation and secretion in response to several agonists including ADP. In addition to the patients mentioned above, who have a defect in the $P2T_{AC}$ ($P2Y_{12}$) ADP receptor, one patient has been briefly described[49] with a defect in the P2Y1 platelet receptor which is coupled to ADP induced calcium mobilization. This patient also had impaired platelet aggregation in response to ADP and other agonists. P2Y1-deficient mice have been reported to have decreased platelet aggregation and resistance to thromboembolism[50,51], with a prolonged bleeding time[50]. Lastly, Oury et al.[52] have recently reported a patient with a selective impairment of the ADP-induced aggregation associated with a dominant negative P2X1 receptor due to deletion of a single leucine residue in the second transmembrane domain. P2X1 receptor is an ATP-gated ion channel, and the association of a bleeding disorder with alterations in this receptor suggests its hitherto unrecognized physiological role in hemostasis.

Selective impairment in platelet responsiveness to epinephrine

Aggregation responses to epinephrine, mediated by α_2-adrenergic receptors, may be variable even in otherwise normal individuals[53] and it is impaired in some presumably normal individuals. In one study[54], the second wave of aggregation was noted to be absent in 10–15% of normal subjects. Studies in twins suggest that platelet adrenergic receptors are under genetic control[55]. Scrutton et al.[56] reported depressed platelet aggregation response to epinephrine in five apparently normal volunteers and indicated that the defect was familial. However, in four of the index cases platelet responses to other agonists such as collagen, vasopressin, and ADP were also impaired. Rao et al.[57] have described a family whose several members had impaired aggregation and secretion responses only to epinephrine associated with decreased number of platelet α_2-adrenergic receptors. Three of the family members had a history of easy bruising with minimally prolonged bleeding times. Although aggregation response was impaired, epinephrine inhibition of adenylate cyclase was normal indicating that the receptor requirements for these two platelet responses are different. Although other families with an epinephrine defect have been reported,[58] the relationship of the selective epinephrine defect to bleeding manifestations still needs to be defined. It is tantalizing to speculate that, in some of these patients with bleeding symptoms, the isolated impaired aggregation response to epinephrine may be related to the Quebec platelet syndrome[59]. Mice deficient in Gz, a member of the Gi family of

Cambridge Books Online © Cambridge University Press, 2009

G-proteins, have impaired platelet aggregation and ability to inhibit cAMP formation in response to epinephrine, and are resistant to fatal thromboembolism[60].

Defects in GTP-binding protein activation

GTP-binding proteins are a group of heterotrimeric (consisting of α, β and γ subunits) proteins that constitute the link between surface receptors and intracellular enzymes, although the specific Gα subunits mediating different agonist responses may be different[61]. Because of their modulating role as molecular switches, G-proteins constitute an important potential locus for aberrations leading to platelet dysfunction. Convincing evidence for the existence of such a defect has been provided by Gabbeta et al.[62] in a patient with a mild bleeding disorder, abnormal aggregation and secretion in response to a number of agonists, and diminished GTPase activity (a reflection of α-subunit function) on activation. The binding of ^{35}S-GTPγS to platelet membranes was diminished, and there was a selective decrease in platelet membrane Gαq with normal levels of Gα_i, Gα_{12}, Gα_{13} and Gα_z. This patient was found to have abnormalities in other downstream signalling events: Ca^{2+} mobilization[63], and release of arachidonic acid from phospholipids on platelet activation[64] despite presence of normal levels of phospholipase A2[62]. Recent studies suggest that this patient has a hematopoietic-lineage restricted defect in Gαq gene expression, characterized by decreased Gαq mRNA and protein levels in platelets but not in neutrophils[64a]. These findings have been corroborated by essentially identical abnormal platelet responses in the Gαq deficient 'knockout' mice[65]. Impaired G-protein activation has also been observed in patients with TxA_2 receptor defect[17,18].

Defects in phospholipase C activation, calcium mobilization, pleckstrin phosphorylation

Several reports have documented patients with a relatively mild bleeding diathesis, and impaired aggregation and dense granule secretion, even though their platelets have normal granule stores and synthesize substantial amounts of TxA_2[66–68]. These heterogenous patients have abnormalities in aggregation and secretion particularly in response to weaker agonists (ADP, epinephrine, PAF); the responses to relatively stronger agonists such as arachidonate and high concentrations of collagen are often normal. Such patients are far more common than those with the storage pool deficiency or defects in TxA_2 synthesis. Lages and Weiss[66] have described eight such patients who had decreased initial rates and extents of aggregation in response to ADP, epinephrine, and U44069; they postulated defects in early platelet activation events to explain the abnormal responses. They subsequently demonstrated a defect in phosphatidylinositol hydrolysis and phosphatidic acid formation[14], and in pleckstrin phosphorylation[69] in one patient.

Koike et al.[67] described platelet dysfunction in 12 patients with the behavioral disorder attention deficit disorder (ADD) and a mild bleeding disorder characterized predominantly by easy bruising. Platelet aggregation and ^{14}C-serotonin secretion responses during stimulation of platelet-rich plasma with ADP, epinephrine, and collagen, revealed only minor abnormalities. In contrast, the aggregation responses of gel-filtered platelets to a divalent cationophore A23187 were markedly impaired in ADD patients. Upon stimulation with A23187 and thrombin (at low concentrations), dense granule and acid hydrolase secretion was impaired whereas α-granule secretion was normal. The platelet contents of all granule constituents were normal, thereby excluding a SPD, and platelet TxA_2 production was intact. The ADD patients and those indicated above[66–68] attest to the existence of a group of patients who have platelet dysfunction despite the presence of normal granule stores and normal TxA_2 synthesis; such patients have been referred to as having primary secretion defects[70].

Platelet receptor ligation results in a rise in cytoplasmic ionized Ca^{2+} concentration which is an early response to platelet stimulation. Attention has, therefore, been focused on this process to explain the impaired platelet aggregation and secretion. In several patients, defects in calcium mobilization have been proposed based on impaired platelet responses to the calcium ionophore (A23187)[10,70]; however, this evidence is indirect, at best. Direct evidence has been provided that some of these patients indeed, have impaired Ca^{2+} mobilization upon platelet activation[63,68,71]. Detailed studies in two related patients[71] with impaired aggregation and secretion responses revealed that resting cytoplasmic Ca^{2+} concentration was normal, but the peak Ca^{2+} concentrations following activation with ADP, collagen, PAF or thrombin were diminished; both the release of Ca^{2+} from intracellular stores and the influx of extracellular Ca^{2+}[63] were decreased. Formation of $InsP_3$, the key intracellular mediator of Ca^{2+} release, as well as diacylglycerol formation and pleckstrin phosphorylation, were diminished upon platelet activation[72], indicating a defect in PLC activation. Human platelets contain at least seven PLC isozymes in the quantitative order PLC-γ2 > PLC-β2 > PLC-β3 > PLC-β1 > PLC-γ1 > PLC-δ1 > PLC-β4[73]. Studies in one of these patients with impaired PLC activation revealed a selective decrease in PLC-β2 isozyme with

Cambridge Books Online © Cambridge University Press, 2009

normal levels of other PLC isoforms[73]. Recent studies show that the decreased platelet PLC-β2 protein levels are associated with diminished platelet PLC-β2 mRNA levels suggesting a defect in gene regulation[74]. Moreover, PLC-β2 protein and mRNA levels in neutrophils were found to be normal suggesting that the defect is hematopoietic lineage restricted. PLC-β isozymes are activated by G-protein mediated pathways while PLC-γ isozymes are activated by tyrosine kinase-dependent mechanisms[75,76]; the relative importance of the various PLC isozymes in normal platelet responses remains unknown. The platelet dysfunction in this patient indicates that PLC-β2, the predominant G-protein linked PLC isozyme in platelets, plays a major role in platelet activation responses involved in hemostasis. In line with the above findings in platelets, agonist-induced calcium mobilization is diminished in neutrophils of knockout mice deficient in PLC- β2[77].

Other studies have also provided evidence for abnormalities in signal transduction pathways in patients with diminished platelet aggregation and secretion responses. Defects in phosphatidylinositol metabolism and protein phosphorylation have been described in such patients[14,17,69,78–80]. Holmsen et al.[78] described a patient with abnormalities in platelet aggregation and dense granule secretion who had impaired phosphoinositide hydrolysis and release of free arachidonic acid from phospholipids on thrombin activation. This patient had significant reduction in membrane GPIIb and GPIIIa as well; however, no studies were performed on Ca^{2+} mobilization or Ins(1,4,5)P_3 production. Another patient has been described with impaired platelet responses and diminished phosphoinositide metabolism in whom the altered stimulus-response coupling has been attributed to abnormal membrane phospholipid composition[79]. Fuse et al.[17] have reported a patient with a mild bleeding disorder whose platelets had impaired aggregation, secretion, $InsP_3$ formation, and Ca^{2+} mobilization in response to a TxA_2 mimetic (STA_2) associated with normal TxA_2 formation. Interestingly, GTPase activity upon activation with STA_2 was also impaired leading to the conclusion that the platelets had an abnormality in coupling between TxA_2 receptor and PLC. In the patient described by Mitsui et al.[80], the abnormal platelet aggregation was associated with decreased TxA_2-induced $InsP_3$ formation but with normal GTPase activity on TxA_2 activation and normal platelet TxA_2 receptors (including their cDNA sequence), suggesting that the abnormality in PLC activity was downstream of the surface receptor. In an analysis of five patients with absent aggregation in response to TxA_2, (but with abnormal responses to ADP and collagen), Fuse et al.[20] found evidence for a defect in the TxA_2 receptor in three patients; in the other two patients TxA_2-induced GTPase activity, IP3 formation and Ca^{2+} mobilization were normal, suggesting a primary abnormality distal to the TxA_2 receptor and unrelated to PLC activation. Overall, these patients provide evidence for aberrations in the signal transduction pathways in patients with diminished platelet aggregation and secretion responses.

Yang et al.[68] have summarized detailed studies on signalling mechanisms in eight patients with abnormal aggregation and secretion in response to several different surface receptor-mediated agonists despite presence of normal dense granule contents. Both protein kinase C induced pleckstrin phosphorylation and cytoplasmic Ca^{2+} mobilization play a major role in secretion on activation[81]. Receptor-mediated Ca^{2+} mobilization and/or pleckstrin phosphorylation were abnormal in seven of the eight patients. It was postulated that combined platelet activation with a direct PKC activator DIC_8 (1,2, dioctonoyl-sn-glycerol) and ionophore A23187, which possibly bypass two major intracellular mediators (inositol trisphosphate, diacylglycerol), may induce normal dense granule secretion in these patients with diminished secretion on activation with receptor mediated agonists. Platelet activation with a combination of ADP with DIC_8 or A23187 improved secretion in four patients. However, combination of DIC_8 and A23187 induced normal secretion in platelet-rich plasma in all patients. These studies indicate that the ultimate process of exocytosis or secretion *per se* is intact in these patients and impaired secretion results from abnormalities in early signal transduction events.

Protein phosphorylation by tyrosine kinases (members of the Src-kinase family, focal adhesion kinase (FAK) family, pp72[syk], and Janus (JAK) kinase family) play an important role in platelet signal transduction[82]. In thrombasthenia[83,84] and the Scott syndrome[85], tyrosine phosphorylation of several proteins is impaired on platelet activation. In these disorders, this defect is a result of the primary abnormality in the GPIIb-IIIa complex and in phospholipid scrambling, respectively[82,84,85]. Interestingly, in patients with the thrombocytopenia with absent radii (TAR) syndrome thrombopoietin-induced tyrosine phosphorylation has been reported to be markedly abnormal[86].

Signal transduction defects and activation of GPIIb–IIIa

Platelet activation induced conformational change in the GPIIb–IIIa complex and fibrinogen-binding to platelets, is a signal transduction dependent process, and has been linked to pleckstrin phosphorylation[87,88]. Therefore, it is likely that abnormalities in signalling mechanisms would

Cambridge Books Online © Cambridge University Press, 2009

impair activation of GPIIb–IIIa, a prerequisite for aggregation. Evidence for this is provided by the report[89] of a patient with markedly abnormal platelet aggregation and receptor activated pleckstrin phosphorylation who had decreased activation of the platelet GPIIb–IIIa complexes despite the presence of a normal number of these platelet receptors with intact ligand (fibrinogen) binding capacity. The implication is that defects in upstream signalling events that modulate activation of the GPIIb–IIIa complex may manifest with decreased platelet aggregation on exposure to receptor-mediated agonists. A similar abnormality in the activation of GPIIb–IIIa has also been observed in the patient with Gαq deficiency[62] attesting to the role of the G-protein-mediated signalling mechanisms in modulating GPIIb–IIIa on activation (Fig. 44.2). Such a defect in GPIIb–IIIa activation, secondary to abnormalities in upstream signal transduction events may be a more common mechanism for blunted aggregation (particularly primary wave) than specific defects in the GPIIb–IIIa complex *per se*[90], and may explain the diminished initial aggregation responses noted by Lages and Weiss[66] in several patients.

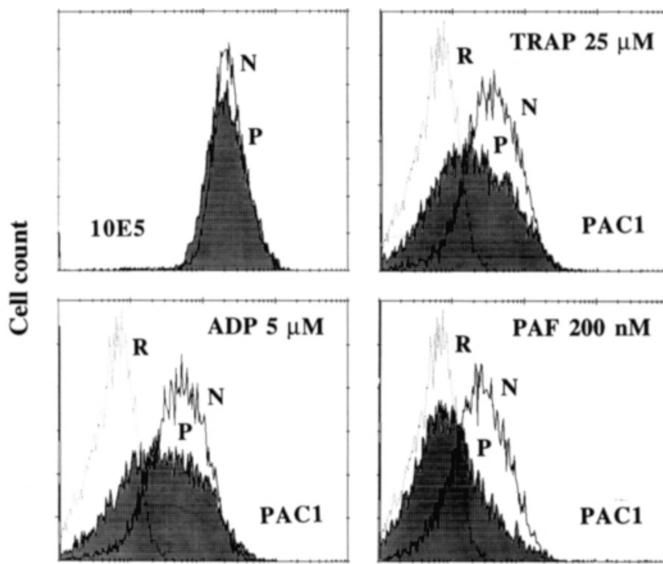


Fig. 44.2. Impaired receptor-mediated activation of GPIIb–IIIa complex on platelets in a patient with Gαq deficiency. Shown is the flow cytometry analysis of GPIIb–IIIa complex on platelets from a normal donor (N) and patient (P) in the resting state (R) and after activation. Top left panel: Binding of monoclonal antibody 10E5 which recognizes both activated and resting forms of GPIIb–IIIa. This panel shows that the GPIIb–IIIa complexes on platelets from the patient and normal are comparable. Top right and lower panels: Binding of antibody PAC-1 which binds only to the activated form of GPIIb–IIIa after activation with thrombin receptor activating peptide (TRAP, SFLLRN), ADP and platelet activating factor. These studies show that despite the presence of a normal complement of GPIIb–IIIa receptors on the patient's platelets, their activation is diminished as revealed by decreased binding of PAC-1. Antibody binding to platelets was measured as fluorescence intensity shown on abscissa and platelet count is on the ordinate. (Reprinted with permission[62].)

Abnormalities in platelet arachidonic acid pathways and thromboxane A_2 production

Platelet activation results in the liberation of arachidonic acid from phospholipids and its subsequent conversion to TxA_2, which forms an important positive feedback enhancing the activation process. TxA_2 synthesis is required for dense granule secretion during stimulation of platelet-rich plasma with ADP, epinephrine, and low concentrations of collagen and thrombin. In general, most patients with defects in the platelet arachidonic acid pathways have had mild to moderate bleeding manifestations.

Defects in the liberation of arachidonic acid from phospholipids

Mobilization of free arachidonic acid from membrane-bound phospholipids PLA_2, a Ca^{2+} dependent enzyme, constitutes the initial and rate-limiting step in TxA_2 synthesis. Four patients have been described[64] with abnormal aggregation and secretion associated with impaired liberation of arachidonic acid from membrane phospholipids during platelet stimulation. In these patients the dense granule contents were normal. Platelet TxA_2 production, measured using a radioimmunoassay, was diminished during stimulation with ADP and thrombin but was normal with free arachidonic acid. In ^{3}H-arachidonic acid labelled platelets, thrombin-induced mobilization of free arachidonic acid from phospholipids was impaired in these patients. Subsequent studies in one of the patients showed that the platelet PLA_2 levels (both membrane and cytosolic) were normal but agonist-induced Ca^{2+} mobilization[63] and G-protein activation[62] were decreased. The impaired arachidonate liberation in this patient is likely secondary to the defect in Ca^{2+} mobilization, preventing full expression of PLA_2 activity. Other reports have also documented patients with an impaired release of arachidonic acid[78,91,92]. In one study[78], the platelets were deficient in GPIIb–IIIa as well; while in another[92], the patient platelets had SPD in addition to diminished platelet uptake of ^{14}C-arachidonic acid and PLA_2 activity.

Cambridge Books Online © Cambridge University Press, 2009

Deficiencies of cyclooxygenase and thromboxane synthase

In 1975, Malmsten et al.[93] reported platelet cyclooxygenase deficiency in a patient with a mild bleeding disorder and impaired aggregation responses to ADP, epinephrine, collagen and arachidonic acid but with normal response to PGG_2. Subsequently, several other patients have been described with a similar defect in TxA_2 synthesis[94–99]. Because cyclooxygenase mediates the endothelial PGI_2 production, the patient described by Pareti et al.[96] had impaired PGI_2 production as well. Interestingly, this patient with defects in both platelet and vessel wall cyclooxygenase had predominantly bleeding symptoms and not thrombotic events. The patient reported by Rak and Boda[97] had progressive arteriosclerosis as evidenced by cerebrovascular and cardiac events. Using a radioimmunoassay, Roth and Machuga[98] found normal cyclooxygenase levels in five of six patients suspected to have a deficiency, suggesting that these patients may have a functionally abnormal molecule. More recently, three patients have been described[99] with impaired platelet responses and markedly decreased ability to convert arachidonic acid, but not of PGH_2, to TxA_2. Using specific antibodies, the authors demonstrated decreased platelet cyclooxygenase-1 levels in two patients and normal levels in the third; levels of thromboxane synthase were normal in all three. Thus, platelet cyclooxygenase deficiency is manifested either by undetectable enzyme protein levels (Type 1) or as an antigenically detectable but functionally abnormal molecule (Type 2)[99].

Thromboxane synthase deficiency has been described in two patients[100,101]. Another patient has been described[102] with bleeding manifestations, whose platelets had impaired aggregation, dense granule secretion and TxA_2 production upon activation. Liberation of arachidonic acid from phospholipids was normal and TxA_2 synthesis was markedly diminished during stimulation of platelet-rich plasma with thrombin, but substantial TxA_2 production was noted on activation of patient's platelets suspended in a buffer containing no albumin. These findings suggest that the platelets had diminished levels of enzyme activity which could express itself adequately only in the absence of albumin, which binds free arachidonic acid avidly. Although the exact site of the enzyme defect was not elucidated, these studies reflect the modulating role of albumin on platelet arachidonate metabolism.

Defects in cytoskeletal assembly

The Wiskott–Aldrich syndrome (WAS) is an X-linked inherited disorder affecting T-lymphocytes and platelets and characterized by thrombocytopenia, eczema, and immunodeficiency. The platelets are smaller than normal with a shortened survival, and several platelet abnormalities have been reported including dense granule SPD[103], deficiencies of GPIb and GPIa, impaired aggregation responses and abnormalities in platelet energy metabolism[103–106]. WAS and the related X-linked thrombocytopenia (XLT) arise from mutations of the X-chromosome gene (location Xp11.22) called WASP which encodes a novel intracellular proline-rich 53-kD protein of 502 amino acids[103,107]. This multifaceted WASP protein appears to constitute a link between the cytoskeleton and signal transduction pathways. WASP has been shown to bind to $p47^{nck}$, a SH3-containing adapter protein, and to active GTP-complexed form of Cdc42, a member of the small GTP-hydrolyzing proteins (GTPases) which is involved in actin remodelling[103,108,109]. WASP has an actin-binding region at the carboxyl end, and an amino terminal pleckstrin homology (PH) domain which plays a role in the binding of the protein to phospholipids (PIP_2). Overall, WASP appears to be a key cytoplasmic regulator of cytoskeletal assembly in the affected WAS cells leading to the unifying concept of WAS as a cytoskeletal disease[103,109]. However, specific abnormalities in platelet cytoskeletal assembly state need to be documented in WAS. In terms of therapy, patients with WAS respond to splenectomy with an increase in platelet number and size and WAS has been successfully managed with bone marrow transplantation[103,110–112]. Serious infections remain a major issue in individuals who undergo splenectomy[111,112].

Miscellaneous disorders associated with platelet function defects

Platelet function abnormalities have been reported in inherited connective tissue disorders such as osteogenesis imperfecta, the Ehlers–Danlos syndrome, and the Marfan's syndrome[113–116] which are associated with bleeding manifestations more likely due to the underlying connective tissue defect rather than the platelet dysfunction. Abnormal platelet responses have been reported in patients with hexokinase deficiency[117], glucose-6 phosphatase deficiency (glycogen storage disease, type I)[118,119], Epstein's syndrome characterized by thrombocytopenia, hereditary nephritis and nerve deafness[120,121], and Down's syndrome[122]. In glucose-6 phosphatase deficiency the platelet abnormalities were reversed following total parenteral nutrition in these patients for 10–12 days[118,119] indicating that the platelets may be intrinsically normal. The May–Hegglin anomaly is characterized by giant platelets,

Cambridge Books Online © Cambridge University Press, 2009

thrombocytopenia and basophilic granulocyte inclusions. Some patients with this anomaly have platelet function and ultrastructural abnormalities[123,124]. Despite the large platelet size, the surface membrane glycoproteins appear to be normal[125]. In all of the disorders described here, the precise mechanisms leading to the impaired platelet responses are poorly understood.

Relative frequency of various platelet abnormalities

Thrombasthenia, the Bernard–Soulier syndrome and afibrinogenemia are rare disorders. It is generally considered that vWD is the most common congenital platelet function disorder, although the severe forms are rare. Patients currently classified in the heterogeneous category of abnormalities in platelet secretion and signal transduction are probably the most frequently encountered inherited platelet defects. In our experience, the SPD is present in less than 10–15% of patients with congenital platelet defects. Abnormalities in TxA_2 production occur in about 10–20% of patients. A large proportion of the remaining patients with abnormal aggregation and secretion responses demonstrate adequate dense granule stores and produce substantial amounts of TxA_2. They may have defects in the early signalling mechanisms. In this highly heterogeneous group, the underlying mechanisms still need to be established.

Therapy of patients with congenital platelet function disorders

The mainstays of therapy of patients with vWD and afibrinogenemia during bleeding episodes and surgical procedures are methods aimed at elevating the deficient factor levels in plasma. Platelet transfusions and 1-desamino-8D-arginine vasopressin (DDAVP) are the main therapy of patients with inherited platelet defects. Because of the wide disparity in bleeding manifestations therapeutic approaches need to be individualized. Platelet transfusions are effective in controlling the bleeding manifestations but come with potential risks associated with blood products, including alloimmunization. Patients with thrombasthenia may develop antibodies[126,127] against GPIIb-IIIa that compromise the efficacy of subsequent platelet transfusions. A viable alternative to platelet transfusions is intravenous administration of DDAVP which shortens the bleeding time in a substantial number of patients with platelet function defects[128–131]. This response appears to be dependent on the defects leading to the platelet dysfunction[128,130,131]. Most patients with thrombasthenia have not responded to DDAVP infusion with a shortening of the bleeding time[128,130–132] with exceptions[133]. However, it is unknown whether DDAVP improves hemostasis in these patients despite a lack of shortening of the bleeding time. Responses in patients with SPD have been variable with a shortening of the bleeding time in some patients[131,134,135] but not others[128,130]. A substantial number of patients with the primary secretion defects appear to respond to DDAVP with a shortening of the bleeding time[130]. In uncontrolled studies it has been feasible to manage selected patients with congenital platelet defects undergoing surgical procedures with DDAVP alone[128,130]. However, this approach needs to be individualized based on the nature of the surgery and the intensity of bleeding symptoms, and platelet transfusions need to be readily available for use in the event of excess hemorrhage. The mechanisms by which DDAVP enhances hemostasis in patients with platelet defects are unclear[128,129]. Its administration induces a rise in plasma vWF, FVIII and tissue plasminogen activator. The abnormal in vitro platelet aggregation or secretion responses in patients with platelet defects are not corrected by DDAVP[130]. Enhancement of aggregation responses by DDAVP or a direct stimulatory effect has been noted in some studies[136] but not others[137,138].

The other approaches that have been utilized to improve hemostasis in patients with inherited platelet defects include a short 3–4 day course of prednisone (20–50 mg)[139] and the administration of antifibrinolytic agents epsilonaminocaproic acid or tranexamic acid which have been successfully used in patients with coagulation disorders[129,140,141]. Although allogeneic bone marrow transplantation has been successfully performed with complete correction in patients with thrombasthenia[142] and the Wiskott–Aldrich syndrome[103,110], such a drastic therapy is rarely required in patients with congenital platelet function disorders.

Acknowledgements

This work was supported by grant R01 HL056724 from NIH-NHLBI and a grant-in-aid from the March of Dimes Birth Defects Foundation. The excellent secretarial assistance of Ms. JoAnn Hamilton is gratefully acknowledged.

Cambridge Books Online © Cambridge University Press, 2009

References

1 Rao AK. Congenital disorders of platelet function: disorders of signal transduction and secretion. *Am J Med Sci* 1998; 316: 69–76.

2 Rao AK, Gabbeta J. Congenital disorders of platelet signal transduction. *Arterioscler Thromb Vasc Biol* 2000; 20: 285–289.

3 Nichols WC, Ginsberg D. von Willebrand's disease. *Medicine* 1997; 76: 1–20.

4 Lopez JA, Andrews RK, Afshar-Kharghan V, Berndt MC. Bernard–Soulier syndrome. *Blood* 1998; 91: 4397–4418.

5 George JN, Nurden AT. Inherited disorders of the platelet membrane: Glanzmann thrombasthenia, Bernard–Soulier syndrome, and other disorders. In: Colman RW, Hirsh J, Marder VJ, Salzman EW, eds. *Hemostasis and thrombosis. Basic Principles and clinical practice.* Philadelphia: J.B. Lippincott; 1994: 652–671.

6 Weiss HJ. Scott syndrome: a disorder of platelet coagulant activity. *Semin Hematol* 1994; 31: 312–319.

7 Weiss HJ. Inherited abnormalities of platelet granules and signal transduction. In: Colman RW, Hirsh J, Marder VJ, Salzman DW, eds. *Hemostasis and thrombosis, basic principles and clinical practice.* 3rd edn. Philadelphia, PA: Lippincott; 1994.

8 Hayward CPM. Inherited disorders of platelet alpha-granules. *Platelets* 1997; 8: 197–209.

9 Pelczar-Wissner CJ, McDonald EG, Sussman, II. Absence of platelet activating factor (PAF) mediated platelet aggregation: a new platelet defect. *Am J Hematol* 1984; 16: 419–422.

10 Lages B, Malmsten C, Weiss HJ, Samuelson B. Impaired platelet response to thromboxane-A_2 and defective calcium mobilization in a patient with a bleeding disorder. *Blood* 1981; 57: 545–552.

11 Samama M, Lecrubier C, Conard J et al. Constitutional thrombocytopathy with subnormal response to thromboxane A_2. *Br J Haematol* 1981; 48: 293–303.

12 Wu KK, Le Breton GC, Thai HH, Chen YC. Abnormal platelet response to thromboxane A_2. *J Clin Invest* 1981; 67: 1801–1804.

13 Rao AK, Willis J, Holmsen H. A major role of ADP in 'thromboxane transfer' experiments. Studies in patients with platelet secretion defects. *J Lab Clin Med* 1984; 104: 116–126.

14 Lages B, Weiss HJ. Impairment of phosphatidylinositol metabolism in a patient with a bleeding disorder associated with defects of initial platelet responses. *Thromb Haemost* 1988; 59: 175–179.

15 Hirata T, Kakizuka A, Ushikubi F, Fuse I, Okuma M, Narumiya S. Arg60 to Leu mutation of the human thromboxane A2 receptor in a dominantly inherited bleeding disorder. *J Clin Invest* 1994; 94: 1662–1667.

16 Ushikubi F, Okuma M, Kanaji K et al. Hemorrhagic thrombocytopathy with platelet thromboxane A2 receptor abnormality: defective signal transduction with normal binding activity. *Thromb Haemost* 1987; 57: 158–164.

17 Fuse I, Mito M, Hattori A et al. Defective signal transduction induced by thromboxane A2 in a patient with a mild bleeding disorder: impaired phospholipase C activation despite normal phospholipase A2 activation. *Blood* 1993; 81: 994–1000.

18 Ushikubi F, Ishibashi T, Narumiya S, Okuma M. Analysis of the defective signal transduction mechanism through the platelet thromboxane A_2 receptor in a patient with polycythemia vera. *Thromb Haemost* 1992; 67: 144–146.

19 Armstrong RA, Jones RL, Peesapati V, Will SG, Wilson NH. Competitive antagonism at thromboxane receptors in human platelets. *Br J Pharmacol* 1985; 84: 595–607.

20 Fuse I, Hattori A, Mito M et al. Pathogenetic analysis of five cases with a platelet disorder characterized by the absence of thromboxane A2 (TXA2)-induced platelet aggregation in spite of normal TXA2 binding activity. *Thromb Haemost* 1996; 76: 1080–1085.

21 Thomas DW, Mannon RB, Mannon PJ et al. Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2. *J Clin Invest* 1998; 102: 1994–2001.

22 Nieuwenhuis HK, Akkerman JWN, Houdijk WPM, Sixma JJ. Human blood platelets showing no response to collagen fail to express surface glycoprotein Ia. *Nature* (London) 1985; 318: 470–472.

23 Nieuwenhuis HK, Sakariassen KS, Houdijk WPM, Nievelstein PFEM, Sixma JJ. Deficiency of platelet membrane glycoprotein Ia associated with a decreased platelet adhesion to subendothelium: a defect in platelet spreading. *Blood* 1986; 68: 692–695.

24 Kehrel B, Balleisen L, Kokott R et al. Deficiency of intact thrombospondin and membrane glycoprotein Ia in platelets with defective collagen-induced aggregation and spontaneous loss of disorder. *Blood* 1988; 71: 1074–1078.

25 Moroi M, Jung SM, Okuma M, Shinmyozu K. A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion. *J Clin Invest* 1989; 84: 1440–1445.

26 Ryo R, Yoshida A, Sugano W et al. Deficiency of P62, a putative collagen receptor, in platelets from a patient with defective collagen-induced platelet aggregation. *Am J Hemat* 1992; 38: 25–31.

27 Arai M, Yamamoto N, Moroi M, Akamatsu N, Fukutake K, Tanoue K. Platelets with 10% of the normal amount of glycoprotein VI have an impaired response to collagen that results in a mild bleeding tendency [published erratum appears in *Br J Haematol* 1995 Apr; 89(4): 952]. *Br J Haematol* 1995; 89: 124–130.

28 Ichinohe T, Takayama H, Ezumi Y et al. Collagen-stimulated activation of Syk but not c-Src is severely compromised in human platelets lacking membrane glycoprotein VI. *J Biol Chem* 1997; 272: 63–68.

29 Watson S, Berlanga O, Best D, Frampton J. Update on collagen receptor interactions in platelets: is the two-state model still valid? *Platelets* 2000; 11: 252–258.

30 Tandon NN, Kralisz U, Jamieson GA. Identification of glyco-

Cambridge Books Online © Cambridge University Press, 2009

protein IV (CD36) as a primary receptor for platelet–collagen adhesion. *J Biol Chem* 1989; 264: 7576–7583.
31 Diaz-Ricart M, Tandon NN, Carretero M, Ordinas A, Bastida E, Jamieson GA. Platelets lacking functional CD36 (glycoprotein IV) show reduced adhesion to collagen in flowing whole blood. *Blood* 1993; 82: 491–496.
32 Yamamoto N, Ikeda H, Tandon NN et al. A platelet membrane glycoprotein (GP) deficiency in healthy blood donors: Nak^{a-} platelets lack detectable GPIV (CD36). *Blood* 1990; 76: 1698–1703.
33 Daniel JL, Dangelmaier C, Strouse R, Smith JB. Collagen induces normal signal transduction in platelets deficient in CD36 (platelet glycoprotein IV). *Thromb Haemost* 1994; 71: 353–356.
34 Kehrel B, Wierwille S, Clemetson KJ et al. Glycoprotein VI is a major collagen receptor for platelet activation: it recognizes the platelet-activating quaternary structure of collagen, whereas CD36, glycoprotein IIb/IIIa, and von Willebrand factor do not. *Blood* 1998; 91: 491–499.
35 Saelman EUM, Kehrel B, Hese KM, deGroot PG, Sixma JJ, Niewenhuis HK. Platelet adhesion to collagen and endothelial cell matrix under flow conditions is not dependent on platelet glycoprotein IV. *Blood* 1994; 83: 3240–3244.
36 Kashiwagi H, Honda S, Tomiyama Y et al. A novel polymorphism in glycoprotein IV (replacement of proline-90 by serine) predominates in subjects with platelet GPIV deficiency. *Thromb Haemost* 1993; 69: 481–484.
37 Lipsky R, Sobieski DA, Tandon NN, Herman J, Ikeda H, Jamieson GA. Detection of GPIV (CD36) mRNA in Nak^{a-} platelets. *Thromb Haemost* 1991; 65: 456–457.
38 Kunapuli SP. Multiple P2 receptor subtypes on platelets: a new interpretation of their function. *Trends Pharmacol Sci* 1998; 19: 391–394.
39 Gachet C, Cattaneo M, Ohlmann P et al. Purinoceptors on blood platelets: further pharmacological and clinical evidence to suggest the presence of two ADP receptors. *Br J Haematol* 1995; 91: 434–444.
40 Gachet C, Hechler B, Leon C et al. Activation of ADP receptors and platelet function. *Thromb Haemost* 1997; 78: 271–275.
41 Cattaneo M, Lecchi A, Randi AM, McGregor JL, Mannucci PM. Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. *Blood* 1992; 80: 2787–2796.
42 Nurden P, Savi P, Heilmann E et al. An inherited bleeding disorder linked to a defective interaction between ADP and its receptor on platelets. Its influence on glycoprotein IIb-IIIa complex function. *J Clin Invest* 1995; 95: 1612–1622.
43 Levy-Toledano S, Maclouf J, Rosa JP et al. Abnormal tyrosine phosphorylation linked to a defective interaction between ADP and its receptor on platelets. *Thromb Haemost* 1998; 80: 463–468.
44 Hollopeter G, Jantzen HM, Vincent D et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* 2001, 409: 202–207.
45 Humbert M, Nurden P, Bihour C et al. Ultrastructural studies of platelet aggregates from human subjects receiving clopidogrel and from a patient with an inherited defect of an ADP-dependent pathway of platelet activation. *Arterioscler Thromb Vasc Biol* 1996; 16: 1532–1543.
46 Cattaneo M, Lombardi R, Zighetti ML, Gachet C, Ohlmann P, Cazenave J. Deficiency of (^{33})P-2MeS-ADP binding sites on platelets with secretion defect, normal granule stores and normal thromboxane A_2 production. *Thromb Haemost* 1997; 77: 986–990.
47 Cattaneo M, Zighetti ML, Lombardi R et al. Platelets for a patient heterozygous for the deficiency of 2MeS-ADP binding sites have a secretion defect. *Eur J Clin Invest* 1997; 27: A23.
48 Cattaneo M, Lecchi A, Lombardi R, Gachet C, Zighetti ML. Platelets from a patient heterozygous for the defect of P2(CYC) receptors for ADP have a secretion defect despite normal thromboxane A(2) production and normal granule stores: further evidence that some cases of platelet 'Primary secretion defect' are heterozygous for a defect of P2(CYC) receptors. *Arterioscler Thromb Vasc Biol* 2000; 20: E101–E106.
49 Oury C, Toth-Zsamboki E, Van Geet C et al. A natural dominant negative P2X1 receptor due to deletion of a single amino acid residue. *J Biol Chem* 2000, 275: 2261–2264.
50 Fabre JE, Nguyen M, Latour A et al. Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y1-deficient mice. *Nat Med* 1999; 5: 1199–1202.
51 Leon C, Hechler B, Freund M et al. Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. *J Clin Invest* 1999; 104: 1731–1737.
52 Oury C, Toth-Zsamboki E, Van Geet C et al. A natural dominant negative P2X1 receptor due to deletion of a single amino acid residue. *J Biol Chem* 2000; 275: 22611–22614.
53 OBrien JR. Variability in the aggregation of human platelets by adrenaline. *Nature* 1964; 202: 1188.
54 Weiss HJ, Lages B. The response of platelets to epinephrine in storage pool deficiency – evidence pertaining to the role of adenosine diphosphate in mediating primary and secondary aggregation. *Blood* 1988; 72: 1717–1725.
55 Propping P, Friedl W. Genetic control of adrenergic receptors on human platelets. A twin study. *Hum Genet* 1983; 64: 105.
56 Scrutton MC, Clare KA, Hutton RA et al. Depressed responsiveness to adrenaline in platelets from apparently normal human donors: a familial trait. *Br J Haematol* 1981; 49: 303.
57 Rao AK, Willis J, Kowalska MA, Wachtfogel Y, Colman RW. Differential requirements for epinephrine induced platelet aggregation and inhibition of adenylate cyclase. Studies in familial $alpha_2$-adrenergic receptor defect. *Blood* 1988; 71: 494–501.
58 Tamponi G, Pannocchia A, Arduino C et al. Congenital deficiency of alpha-2-adrenoreceptors on human platelets: description of two cases. *Thromb Haemost* 1987; 58: 1012.
59 Hayward CPM, Rivard GE, Kane WH. An autosomal dominant, qualitative platelet disorder associated with multimerin deficiency, abnormalities in platelet factor V, thrombospondin, von Willebrand factor, and fibrinogen, and an epinephrine aggregation defect. *Blood* 1996; 87: 4967–4978.

Cambridge Books Online © Cambridge University Press, 2009

60 Yang J, Wu J, Kowalska MA et al. Loss of signaling through the G protein, gz, results in abnormal platelet activation and altered responses to psychoactive drugs. *Proc Natl Acad Sci USA* 2000; 97: 9984–9989.
61 Brass LF, Hoxie JA, Manning DR. Signaling through G proteins and G protein-coupled receptors during platelet activation. *Thromb Haemost* 1993; 70: 217–223.
62 Gabbeta J, Yang X, Kowalska MA, Sun L, Dhanasekaran N, Rao AK. Platelet signal transduction defect with Gα subunit dysfunction and diminished Gαq in a patient with abnormal platelet responses. *Proc Natl Acad Sci USA* 1997; 94: 8750–8755.
63 Rao AK, Disa J, Yang X. Concomitant defect in internal release and influx of calcium in patients with congenital platelet dysfunction and impaired agonist-induced calcium mobilization: thromboxane production is not required for internal release of calcium. *J Lab Clin Med* 1993; 121: 52–63.
64 Rao AK, Koike K, Willis J et al. Platelet secretion defect associated with impaired liberation of arachidonic acid and normal myosin light chain phosphorylation. *Blood* 1984; 64: 914–921.
64a Gabbeta J, Vaidyula VR, Dhanasekaran DN, Rao AK. Human platelet Gαq deficiency is associated with decreased Gαq gene expression in platelets but not neutrophils. *Thromb Haemost* (in press), 2001.
65 Offermanns S, Toombs CF, Hu YH, Simon MI. Defective platelet activation in Gaq-deficient mice. *Nature* 1997; 389: 183–186.
66 Lages B, Weiss HJ. Heterogeneous defects of platelet secretion and responses to weak agonists in patients with bleeding disorders. *Br J Haematol* 1988; 68: 53–62.
67 Koike K, Rao AK, Holmsen H, Mueller PS. Platelet secretion defect in patients with the attention deficit disorder and easy bruising. *Blood* 1984; 63: 427–433.
68 Yang X, Sun L, Gabbeta J, Rao AK. Platelet activation with combination of ionophore A23187 and a direct protein kinase C activator induces normal secretion in patients with impaired receptor mediated secretion and abnormal signal transduction. *Thromb Res* 1997; 88: 317–328.
69 Speiser-Ellerton S, Weiss HJ. Studies on platelet protein phosphorylation in patients with impaired responses to platelet agonists. *J Lab Clin Med* 1990; 115: 104–111.
70 Rao AK. Congenital disorders of platelet function. *Hematol/Oncol Clin N Amer* 1990; 4: 65–87.
71 Rao AK, Kowalska MA, Disa J. Impaired cytoplasmic ionized calcium mobilization in inherited platelet secretion defects. *Blood* 1989; 74: 664–672.
72 Yang X, Sun L, Ghosh S, Rao AK. Human platelet signaling defect characterized by impaired production of 1,4,5 inositol triphosphate and phosphatic acid, and diminished pleckstrin phosphorylation. Evidence for defective phospholipase C activation. *Blood* 1996; 88: 1676–1683.
73 Lee SB, Rao AK, Lee K-H, Yang X, Bae YS, Rhee SG. Decreased expression of phospholipase C-β2 isozyme in human platelets with impaired function. *Blood* 1996; 88: 1684–1691.
74 Mao GF, Vaidyula VR, Kunapuli SP, Rao AK. Lineage specific defect in gene expression in human platelet phospholipase C-β2 deficiency. *Blood* (in press), 2001.
75 Rhee SG, Choi KD. Regulation of inositol phospholipid-specific phospholipase C isozymes. *J Biol Chem* 1992; 267: 12393–12396.
76 Lee SB, Rhee SG. Significance of PIP2 hydrolysis and regulation of phospholipase C isozymes. *Curr Opin Cell Biol* 1995; 7: 183–189.
77 Jiang H, Kuang Y, Wu Y, Xie W, Simon MI, Wu D. Roles of phospholipase C β2 in chemoattractant-elicited responses. *Proc Natl Acad Sci USA* 1997; 94: 7971–7975.
78 Holmsen H, Walsh PN, Koike K et al. Familial bleeding disorder associated with deficiencies in platelet signal processing and glycoproteins. *Br J Haematol* 1987; 67: 335–344.
79 Cartwright J, Hampton KK, Macneil S, Colvin BT, Preston FE. A haemorrhagic platelet disorder associated with altered stimulus–response coupling and abnormal membrane phospholipid composition. *Br J Haematol* 1994; 88: 129–136.
80 Mitsui T. Defective signal transduction through the thromboxane A_2 receptor in a patient with a mild bleeding disorder. Deficiency of the inositol 1,4,5-triphosphate formation despite normal G-protein activation. *Thromb Haemost* 1997; 77: 991–995.
81 Nishizuka Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* 1986; 308: 693–698.
82 Jackson SP, Schoenwaelder SM, Yuan Y, Salem HH, Cooray P. Non-receptor protein tyrosine kinases and phosphatases in human platelets. *Thromb Haemost* 1996; 76: 640–650.
83 Ferrell JE, Martin GS. Tyrosine-specific protein phosphorylation is regulated by glycoprotein IIb–IIIa in platelets. *Proc Natl Acad Sci USA* 1989; 86: 2234–2238.
84 Golden A, Brugge JS, Shattil SJ. Role of platelet membrane glycoprotein IIb–IIIa in agonist-induced tyrosine phosphorylation of platelet proteins. *J Cell Biol* 1990; 111: 3117–3127.
85 Dekkers DW, Comfurius P, Vuist WM et al. Impaired Ca2+-induced tyrosine phosphorylation and defective lipid scrambling in erythrocytes from a patient with Scott syndrome: a study using an inhibitor for scramblase that mimics the defect in Scott syndrome. *Blood* 1998; 91: 2133–2138.
86 Ballmaier M, Schulze H, Cremer M, Folman CC, Strauss G, Weite K. Defective c-Mpl signaling in the syndrome of thrombocytopenia with absent radii. *Stem Cells* 1998; 16: 177–184.
87 Shattil SJ, Brass LF. Induction of the fibrinogen receptor on human platelets by intracellular mediators. *J Biol Chem* 1987; 262: 992–1000.
88 van Willigen G, Akkerman JWN. Protein kinase C and cAMP regulate reversible exposure of binding sites for fibrinogen on glycoprotein IIb–IIIa complex of human platelets. *Biochem J* 1991; 273: 115–120.
89 Gabbeta J, Yang X, Sun L, McLane M, Niewiarowski S, Rao AK. Abnormal inside-out signal transduction-dependent activation of GPIIb–IIIa in a patient with impaired pleckstrin phosphorylation. *Blood* 1996; 87: 1368–1376.
90 Gabbeta J, Rao AK. Impaired platelet aggregation and abnor-

Cambridge Books Online © Cambridge University Press, 2009

mal activation of GPIIb–IIIa [letter]. *Thromb Haemost* 1997; 78: 1302.

91 Deykin D, Rittenhouse-Simmons S, Russell PA. Impaired Ca^{2+} mobilization: a possible cause of platelet dysfunction. *Clin Res* 1978; 21: 503a.

92 Rendu F, Breton-Gorius J, Trugnan G et al. Studies on a new variant of the Hermansky-Pudlak syndrome: qualitative, ultrastructural, and functional abnormalities of the platelet-dense bodies associated with a phospholipase A defect. *Am J Hematol* 1978; 4: 387–399.

93 Malmsten C, Hamberg M, Svensson J. Physiological role of an endoperoxide in human platelets: Hemostatic defect due to platelet cyclooxygenase deficiency. *Proc Natl Acad Sci USA* 1975; 72: 1446–1450.

94 Horellou MH, Lecompte T, Lecrubier C et al. Familial and constitutional bleeding disorder due to platelet cyclooxygenase deficiency. *Am J Hematol* 1983; 14: 1–9.

95 Lagarde M, Byron PA, Vargaftig BB et al. Impairment of platelet thromboxane A_2 generation and of the platelet release reaction in two patients with congenital deficiency of platelet cyclooxygenase. *Br J Haematol* 1978; 38: 251–66.

96 Pareti FI, Mannucci PM, D'Angelo A et al. Congenital deficiency of thromboxane and protacyclin. *Lancet* 1980; i: 898–901.

97 Rak K, Boda Z. Hemostatic balance in congenital deficiency of platelet cyclooxygenase. *Lancet* 1980; ii: 44.

98 Roth GJ, Machuga R. Radioimmune assay of human platelet prostaglandin synthetase. *J Lab Clin Med* 1982; 99: 187–196.

99 Matijevic-Aleksic N, McPhedran P, Wu KK. Bleeding disorder due to platelet prostaglandin H synthase-1 (PGHS-1) deficiency. *Br J Haematol* 1996; 92: 212–217.

100 Defryn G, Machin SJ, Carreras LD, Danden MV, Chamone DAF, Vermylen J. Familial bleeding tendency with partial platelet thromboxane synthetase deficiency: reorientation of cyclic endoperoxide metabolism. *Br J Haematol* 1981; 49: 29–41.

101 Mestel F, Oetliker O, Beck E et al. Severe bleeding associated with defective thromboxane synthetase. *Lancet* 1980; 1: 157.

102 Rao AK, Koike K, Day HJ, Smith JB, Holmsen H. Bleeding disorder associated albumin-dependent partial deficiency in platelet thromboxane production. Effect of albumin on arachidonate metabolism in platelets. *Am J Clin Pathol* 1985; 83: 687–696.

103 Remold-ODonnell E, Rosen FS, Kenney DM. Defects in Wiskott–Aldrich syndrome blood cells. *Blood* 1996; 87: 2621–2631.

104 Grottum KA, Hovig T, Holmsen H, Abrahamsen AF, Jeremic M, Seip M. Wiskott–Aldrich syndrome: qualitative platelet defects and short platelet survival. *Br J Haematol* 1969; 17: 373–388.

105 Baldini MG. Nature of the platelet defect in the Wiskott–Aldrich syndrome. *Ann NY Acad Sci* 1972; 201: 437–444.

106 Stormorken H, Hellum B, Egeland T, Abrahamsen TG, Hovig T. X-linked thrombocytopenia and thrombocytopathia: attenuated Wiskott–Aldrich syndrome. Functional and morphological studies of platelets and lymphocytes. *Thromb Haemost* 1991; 65: 300–305.

107 Zhu Q, Watanabe C, Liu T et al. Wiskott–Aldrich syndrome/X-linked thrombocytopenia: WASP gene mutations, protein expression, and phenotype. *Blood* 1997; 90: 2680–2689.

108 Rosen FS. The enigma of the Wiskott–Aldrich syndrome begins to unravel [editorial; comment]. *J Clin Invest* 1996; 97: 2407.

109 Featherstone C. How does one gene cause Wiskott–Aldrich syndrome? *Lancet* 1996; 348: 950.

110 Mullen CA, Anderson KD, Blaese RM. Splenectomy and/or bone marrow transplantation in the management of the Wiskott–Aldrich syndrome: long-term follow-up of 62 cases. *Blood* 1993; 82: 2961–2966.

111 Lum LG, Tubergen DG, Corash L, Blaese RM. Splenectomy in the management of the thrombocytopenia of the Wiskott–Aldrich syndrome. *N Engl J Med* 1980; 302: 892–896.

112 Litzman J, Jones A, Hann I, Chapel H, Strobel S, Morgan G. Intravenous immunoglobulin, splenectomy, and antibiotic prophylaxis in Wiskott–Aldrich syndrome. *Arch Dis Child* 1996; 75: 436–439.

113 Estes JW. Platelet abnormalities in heritable disorders of connective tissue. *Ann NY Acad Sci* 1972; 201: 445–450.

114 Evensen SA, Myhre L, Stormorken H. Haemostatic studies in osteogenesis imperfecta. *Scand J Haematol* 1984; 33: 177.

115 Hathaway WE, Solomons CC, Ott JE. Platelet function and pyrophosphates in osteogenesis imperfecta. *Blood* 1972; 39: 500–509.

116 Onel D, Ulutin SB, Ulutin ON. Platelet defect in a case of Ehlers-Danlos syndrome. *Acta Haematol* 1973; 50: 238–244.

117 Akkerman JWN, Rijksen G, Gorter G et al. Platelet functions and energy metabolism in a patient with hexokinase deficiency. *Blood* 1984; 63: 147–153.

118 Corby DG, Putnam CW, Greene HL. Impaired platelet function in glucose-6-phosphatase deficiency. *J Pediat* 1974; 85: 71–76.

119 Czapek EE, Deykin D, Salzman EW. Platelet dysfunction in glycogen storage disease type I. *Blood* 1973; 41: 235–247.

120 Bernheim J, Dechavanne M, Byron PA et al. Thrombocytopenia, macrothrombocytopathia, nephritis and deafness. *Am J Med* 1976; 61: 145–150.

121 Epstein CJ, Sahud MA, Piel CF et al. Hereditary macrothrombocytopathia, nephritis and deafness. *Am J Med* 1972; 52: 299–310.

122 Boullin DJ, O'Brien RA. Abnormalities of 5-hydroxytryptamine uptake and binding by blood platelets from children with Down's syndrome. *J Physiol* 1971; 212: 287–297.

123 Hamilton RW, Shaikh BS, Ottie JN et al. Platelet function, ultrastructure, and survival in the May–Heggelin anomaly. *Am J Clin Pathol* 1980; 74: 663–668.

124 Lusher JM, Schneider J, Mizukami I et al. The May–Heggelin anomaly: platelet function, ultrastructure and chromosome studies. *Blood* 1968; 32: 950.

125 Coller BS, Zarrabi MH. Platelet membrane studies in the May–Heggelin anomaly. *Blood* 1981; 58: 279–284.

Cambridge Books Online © Cambridge University Press, 2009

126 Degos L, Dautigny A, Brouet JC et al. A molecular defect in thrombasthenic platelets. *J Clin Invest* 1975; 56: 236–240.
127 George JN, Caen JP, Nurden AT. Glanzmann's thrombasthenia: the spectrum of clinical disease. *Blood* 1990; 75: 1383–1395.
128 Mannucci PM. Desmopressin (DDAVP) in the treatment of bleeding disorders; the first 20 years. *Blood* 1997; 90: 2515–2521.
129 Mannucci PM. Hemostatic Drugs. *N Engl J Med* 1998; 339: 245–253.
130 Rao AK, Ghosh S, Sun L et al. Effect of mechanism of platelet dysfunction on response to DDAVP in patients with congenital platelet function defects. A double-blind placebo-controlled trial. *Thromb Haemost* 1995; 74: 1071–1078.
131 Kobrinsky NL, Israels ED, Gerrard JM et al. Shortening of bleeding time by 1-deamino-8-D-arginine vasopressin in various bleeding disorders. *Lancet* 1984; i: 1145–1148.
132 Schulman S, Johnson H, Egberg N, Blombäck M. DDAVP-induced correction of prolonged bleeding time in patients with congenital platelet function defects. *Thromb Res* 1987; 45: 165–174.
133 DiMichele DM, Hathaway WE. Use of DDAVP in inherited and acquired platelet dysfunction. *Am J Hematol* 1990; 33: 39–45.
134 Mannucci PM. Desmopressin (DDAVP) for treatment of disorders of hemostasis. *Progress in Hemostasis and Thrombosis*. Orlando, FL: Grune & Stratton; 1986: 19–44.
135 Nieuwenhuis HK, Sixma JJ. 1–Desamino-8-D-arginine vasopressin (Desmopressin) shortens the bleeding time in storage pool deficiency. *Ann Int Med* 1988; 108: 65–67.
136 Balduini CL, Noris P, Belletti S, Spedini P, Gamba G. In vitro and in vivo effects of desmopressin on platelet function. *Haematologica* 1999; 84: 891–896.
137 Yang X, Disa J, Rao AK. Effect of 1-desamino-8-arginine vasopressin (DDAVP) on human platelets. *Thromb Res* 1990; 59: 809–818.
138 Ghosh S, Rao AK. Lack of effect of 1–desamino-8–D-arginine vasopressin on direct adhesion of platelets to collagen. *Thromb Res* 1993; 70: 417–422.
139 Mielke CH, Jr., Levine PH, Zucker S. Preoperative prednisone therapy in platelet function disorders. *Thromb Res* 1981; 21: 655–662.
140 Berliner S, Horowitz I, Martinowitz U, Brenner B, Seligsohn U. Dental surgery in patients with severe factor XI deficiency without plasma replacement. *Blood Coagul Fibrinol* 1992; 3: 465–468.
141 Sindet-Pedersen S, Ramstrom G, Bernvil S, Blomback M. Hemostatic effect of tranexamic acid mouthwash in anticoagulant-treated patients undergoing oral surgery. *N Engl J Med* 1989; 320: 840–843.
142 Bellucci S, Devergie A, Gluckman E et al. Complete correction of Glanzmann's thrombasthenia by allogeneic bone-marrow transplantation. *Br J Haematol* 1985; 59: 635–641.

Cambridge Books Online © Cambridge University Press, 2009

Acquired platelet function defects

Joel S. Bennett

University of Pennsylvania School of Medicine, Philadelphia, USA

Platelet function can be impaired by medications and by non-hematologic and hematologic disorders (Table 45.1). Although the effect of these drugs and disorders on platelet function is usually mild, it can be substantial when it is associated with other hemostatic disorders.

Medications that affect platelet function

The effect of medications is the most common cause of acquired platelet dysfunction (Table 45.2)[1]. While some medications simply prolong the bleeding time without causing bleeding, others prolong the bleeding time and either cause or exacerbate a bleeding diathesis. Moreover, it is important to recognize that many drugs affect ex vivo and in vitro platelet function but without obvious clinical consequences.

Table 45.1. Acquired qualitative platelet disorders

1. *Drugs that affect platelet function*
2. *Systemic disorders associated with abnormal platelet function*
   - Uremia
   - Antiplatelet antibodies
   - Cardiopulmonary bypass
   - Miscellaneous disorders
     - Liver disease
     - Disseminated intravascular coagulation
     - Bartter syndrome
3. *Hematological disorders associated with abnormal platelet function*
   - Chronic myeloproliferative disorders
   - Leukemias and myelodysplastic syndromes
   - Dysproteinemias
   - Acquired von Willebrand disease

Table 45.2. Drugs that inhibit platelet function

Non-steroidal anti-inflammatory drugs
Aspirin; reversible cyclooxygenase inhibitors such as indomethacin, ibuprofen, sulindac, and naproxen

Antibiotics
Penicillins, cephalosporins, nitrofurantoin, Miconazole

Thienopyridines
Ticlopidine, clopidogrel

GPIIb/IIIa antagonists
Abciximab, tirofiban,eptifibatide

Drugs that affect platelet cAMP levels
Prostacyclin, iloprost, dipyridamole, cilostazol,

Anticoagulants and fibrinolytic agents
Heparin, streptokinase, tissue plasminogen activator, urokinase, ε-aminocaproic acid

Cardiovascular drugs
Nitroglycerin, isosorbide dinitrate, propranolol, nitroprusside, nifedipine, verapamil, diltiazem, quinidine

Volume expanders
Dextran, hydroxyethyl starch

Psychotropic drugs and anesthetics
Psychotropic drugs: imiprimine, amitriptyline, nortryptaline, chlorpromazine, promethazine, flufenazine, trifluoperazine, haloperidol

Anesthetics: dibucaine, tetracaine, metycaine, cyclaine, butacaine, nepercaine, procaine, cocaine, plaquenil, halothane

Chemotherapeutic agents
Mithramycin, daunorubicin, BCNU

Miscellaneous drugs
Serotonin antagonists
Antihistamines
Radiographic contrast agent
Foods and food additives

Aspirin and other non-steroidal anti-inflammatory drugs

Aspirin

Aspirin inhibits platelet function by irreversibly inactivating the enzyme prostaglandin endoperoxide H synthase-1 (PGHS-1, cyclooxygenase-1, COX-1)[2], thereby preventing the conversion of arachidonic acid to the thromboxane synthase substrate prostaglandin H_2. The resulting inability to synthesize thromboxane A_2, a platelet agonist and vasoconstrictor, impairs platelet responses to ADP, epinephrine, arachidonic acid, and to low doses of collagen and thrombin, although responses to higher doses of collagen or thrombin are unaffected[3,4]. A single 100-mg dose of aspirin almost completely inhibits platelet cyclooxygenase, as does a 30 mg dose taken daily for 7–10 days[5]. Aspirin irreversibly inhibits cyclooxygenase in endothelial cells[6], but has no lasting effect on prostacyclin production by these cells because they can synthesize additional enzyme[7,8].

Aspirin is one of the few drugs that prolongs the bleeding time in humans. In normal subjects, aspirin prolongs the bleeding time to no more than 1.2 to 2.0 times the baseline[9,10]. The bleeding time may remain prolonged for 1 to 4 days after aspirin administration has been discontinued and ex vivo platelet aggregation may be abnormal for up to a week, until the affected platelets are replaced by unaffected ones[11].

In normal subjects, aspirin has a minimal effect on hemostasis, although chronic aspirin ingestion has been reported to increase bruising, epistaxis and gastrointesinal blood loss[12]; the latter likely due to a direct effect on the gastric mucosa[13,14]. When aspirin was taken as primary prophylaxis against myocardial infarction, there was a slight increase in hemorrhagic strokes [12]. Aspirin may also increase maternal and neonatal bleeding during parturition [15] and some studies suggest that preoperative aspirin administration increases blood loss following cardiothoracic and general surgery[16–18]. On the other hand, retrospective analysis indicates that aspirin administration does not affect the safety of epidural and spinal anesthesia[19]. Aspirin markedly prolongs the bleeding time and can precipitate hemorrhage in individuals with preexisting hemostatic defects such as von Willebrand disease, hemophilia, warfarin ingestion, uremia, and disorders of platelet function[20–22]. Ethanol has been reported to potentiate the effect of aspirin[23,24], whereas DDAVP can shorten a prolonged bleeding time due to aspirin[25,26].

Other non-steroidal anti-infammatory drugs (NSAIDS)

NSAIDS such as indomethacin, ibuprofen, naproxen, phenylbutazone, and sulfinpyrazone reversibly inhibit platelet cyclooxygenase by competing with arachidonic acid for binding to the active site of the enzyme[2]. Therapeutic doses of NSAIDS may cause a transient prolongation of the bleeding time; however, this is usually not clinically significant[27–29]. Ibuprofen has been given safely to patients with hemophilia A[30,31]. Analgesics such as acetaminophen, sodium or choline salicylate, and narcotics neither inhibit cyclooxygenase nor prolong the bleeding time[30,32,33].

Antibiotics

Large doses of penicillin can impair ex vivo platelet aggregation and secretion, inhibit ristocetin-induced platelet agglutination, and prolong the bleeding time[34–36] by impairing the interaction of agonists and von Willebrand factor (vWf) with the platelet membrane[37,38]. Thus, penicillin may bind to membrane components necessary for platelet adhesion and/or signal transduction. Consistent with this possibility, the relative antiplatelet activity of various penicillins correlates with their lipid solubility and with the inhibitory potency of the isolated side chains[39,40].

The maximal effect of penicillin on platelet function is seen after 1 to 3 days of administration and may persist for several days after the antibiotic is stopped. Although clinically significant bleeding has been reported with carbenicillin, penicillin G, ticarcillin, and nafcillin, it is far less common than prolongation of the bleeding time[34,41]. Patients with coexisting hemostatic defects may be prone to bleeding. A similar pattern of platelet dysfunction has been reported with cephalosporins moxalactam and cefoxitin, but not with ceftizoxime, cefoperazone, and ceftriaxone[34,42,43]. Moxalactam, cefamandole, and cefoperazone also induce hypothrombinemia, either by decreasing the amount of vitamin K synthesized by the intestinal microflora or by directly inhibiting the vitamin K-dependent carboxylase[34,44]. Nitrofurantoin, a structurally unrelated antibiotic, may cause a mild prolongation of the bleeding time and impair platelet aggregation when blood levels of the drug are higher than 20 μM[45]. Miconazole, an antifungal agent, has been shown to inhibit human and rabbit platelet cyclooxygenase in vitro and rabbit platelet cyclooxygenase after intravenous infusion[46].

Thienopyridines

The thienopyridines ticlopidine and clopidogrel inhibit platelet function by irreversibly antagonizing the G_i-

Cambridge Books Online © Cambridge University Press, 2009

coupled platelet ADP receptor $P2Y_{12}$[47–49]. Thus, ADP-induced platelet shape change and calcium transients are unimpaired, whereas the ability of ADP to inhibit PGI_2-stimulated increases in cAMP is inhibited. Both drugs are prodrugs; hepatic metabolism is required to generate the active agents[50]. Thus, although inhibition of platelet aggregation and prolongation of the bleeding time may be seen within 24–48 hours of the first dose, the effects are not maximal for 4–6 days and may last for 4–10 days after the drugs have been discontinued.

Ticlopidine administration is associated with potentially serious hematological complications, including a 2% incidence of neutropenia[50–52], and less commonly, aplastic anemia, thrombotic thrombocytopenic purpura, and thrombocytopenia[53–57]. Results from the CAPRIE trial suggest that these complications are far less common with clopidogrel[58]. However, 20 cases of TTP have been reported since clopidogrel was approved by the US FDA in 1998[59,60].

GPIIb/IIIa receptor antagonists

Three intravenous GPIIb/IIIa antagonists – abciximab, eptifibatide, and tirofiban – have been approved for use in the treatment of acute coronary artery disease and a number of orally-active GPIIb/IIIa antagonists are undergoing development. As would be expected, these drugs predispose to bleeding. In the EPIC trial, 14% of patients given abciximab (a chimeric human-murine anti-GPIIb/IIIa monoclonal antibody Fab fragment) plus heparin and aspirin during percutaneous coronary angioplasty experienced major bleeding compared to 7% of patients receiving only heparin and aspirin[61]. In the subsequent EPILOG trial, in which the dose of heparin was decreased, the incidence of major bleeding in patients receiving abciximab decreased to 2.0% compared to 3.1% in the control group[62], implying that bleeding in patients given GPIIb/IIIa antagonists can be minimized by paying careful attention to other factors that impair hemostasis[63]. Platelet transfusions readily reverse GPIIb/IIIa antagonism by abciximab by decreasing the extent of GPIIb/IIIa blockade. The ability of platelet transfusion to reverse the effects of the other GPIIb/IIIa antagonists is less clear, but these drugs have short half-lives when renal and hepatic function are normal.

Thrombocytopenia has been observed in small numbers of patients given GPIIb/IIIa antagonists[64–67] and must be differentiated from pseudothrombocytopenia that accounts for approximately 30% of the thrombocytopenia observed in patients receiving these drugs[68]. Rarely, acute profound thrombocytopenia with platelet counts below 20 000/μl occurs within 2–4 hours after initiating therapy[69,70]. The pathogenesis of this thrombocytopenia is uncertain, but it readily reverses when the drugs are stopped and may be reversed more rapidly by platelet transfusion[63].

Drugs that affect platelet cyclic nucleotide levels

Increasing the platelet content of cAMP impairs platelet function. Although the pyrimidopyrimidine derivative dipyridamole increases platelet cAMP by inhibiting its degradation by cyclic nucleotide phosphodiesterase (PDE), its utility at clinically achievable concentrations is controversial[71,72]. On other hand, treatment with a sustained release dipyridamole preparation plus aspirin may be beneficial in the secondary prevention of stroke and transient ischemic attack[73]. Cilostazol, a specific inhbitor of PDE3, the most abundant PDE in platelets[74], has been approved for the treatment of peripheral vascular disease[75] and may be useful in the prevention of cardiac stent occlusion[76]. Intravenous infusions of prostaglandin E_1 (PGE_1), prostacyclin, or stable analogues of prostacyclin decrease platelet responsiveness by stimulating platelet adenylyl cyclase[77–79]. Their clinical utility is limited by their short half-life and side effects due to peripheral vasodilatation[77,80]. Nitric oxide and organic nitrates such as nitroglycerin inhibit platelet function in vitro by activating guanylyl cyclase[81]. Their effect on platelet function in vivo is uncertain. High concentrations of caffeine and theophylline also inhibit platelet phosphodiesterases in vitro.

Anticoagulants and fibrinolytic agents

Heparin can impair platelet function by inhibiting the generation of thrombin, a potent platelet agonist, and perhaps by inhibiting thrombin binding to GPIb[82]. Conversely, in vitro studies suggest that heparin can enhance platelet aggregation induced by other platelet agonists[83]. Large doses of heparin can also impair vWf-dependent platelet function, possibly by binding to the heparin-binding domain of vWf[84]. Whether any of these observations contribute to the bleeding complications of heparin therapy is uncertain. Pharmacologic doses of streptokinase, urokinase, and tissue plasminogen activator (t-PA) can affect platelet function[85]. High concentrations of plasmin in vitro cause platelet aggregation[86] and increases in the urinary excretion of thromboxane A_2 metabolites have been observed in patients receiving streptokinase or t-PA, implying that platelet activation had occurred[87,88]. On the other hand, a number of studies suggest that plasmin can inhibit platelet function. For example, high levels of plasmin-generated fibrin(ogen) degradation products

Cambridge Books Online © Cambridge University Press, 2009

impair platelet aggregation[89] and plasmin enzymatically degrades platelet GPIb[90,91], inhibits platelet arachidonic acid metabolism[92], and promotes the disaggregation of platelet aggregates by lysing GPIIb/IIIa bound fibrinogen[93]. Whether these observations are clinically significant is uncertain[94].

Miscellaneous agents

A number of other medications have been reported to affect in vitro platelet function, but this appears to be of little clinical significance. Drugs in this category include the cardiovascular drugs nitroprusside[95,96], nitroglycerine[97], nitric oxide[98–100], propranolol[101,102] and calcium channel blockers such as verapamil, nifedipine, and diltiazem[103,104]. The volume expander dextran can prolong the bleeding time of patients with von Willebrand disease, but this has not been observed in most normal subjects[105–107]. Hydroxyethyl starch, while generally safe, may prolong the bleeding time and predispose to hemorrhage, particularly if it is administered in doses exceeding 20 ml/kg of a 6% solution[108–110]. Antidepressants and phenothiozines can impair platelet aggregation ex vivo, but this is not associated with bleeding[111,112]. The general anesthesic halothane can cause a slight bleeding time prolongation, but this has no adverse effect on surgical hemostasis[113]. Cocaine has been reported to either inhibit platelet function[114,115] or to induce platelet activation[116].

The chemotherapeutic drug mithramycin has been associated with mucocutaneous bleeding, increased bleeding times, and decreased platelet aggregation[117]. An ex vivo defect in platelet secretion and secondary aggregation has been observed in patients receiving autologous bone marrow and high dose chemotherapy with cisplatin, cyclophosphamide, and either BCNU or melphelan[118]. Daunorubicin and BCNU inhibit platelet aggregation and secretion in vitro, but do not induce clinically significant platelet dysfunction[119–121]. Cyclosporine A may enhance ADP-stimulated platelet aggregation ex vivo and in vitro[122–124]. Antihistamines[125], the serotonin antagonist ketanserin[126], and some radiographic contrast agents[127,128] impair platelet aggregation ex vivo by unknown mechanisms.

Diets rich in ω-3 fatty acids cause a slight prolongation of the bleeding time[129] by reducing the platelet content of arachidonic acid and by competing with arachidonic acid for cyclooxygenase[130,131]. Easy bruising noted after eating Chinese food has been attributed to an effect of the black tree fungus[132]. An onion extract can inhibit platelet arachidonic acid metabolism[133]. The garlic component, ajoene, inhibits platelet aggregation[134,135]. Extracts of two commonly used spices, cumin and turmeric, inhibit platelet aggregation and eicosanoid biosynthesis[136].

Systemic disorders associated with abnormal platelet function

Uremia

Bleeding can be a serious complication of acute and chronic renal failure[137], although the frequency of severe, spontaneous hemorrhage decreased with the advent of dialysis. Moreover, experience with percutaneous renal biopsy in several thousand patients with renal disease indicates that their hemostatic defect is usually mild[138,139].

The hemostatic defect in uremia has been attributed to abnormal platelet function[140]. Platelet adhesion is defective in uremia[141–143]. One major factor may be anemia. Lowering the hematocrit causes an ex vivo platelet adhesion defect that can be corrected by increasing the hematocrit to $\geq$30%[141]. In uremic patients, increasing the hematocrit to 27–32% by red blood cell transfusion or erythropoietin administration either partially or completely normalizes the bleeding time[140,144–148]. However, the effect of anemia on the bleeding time is not unique to uremia. In normal subjects, the bleeding time correlates with the hematocrit, even though both sets of values are in the normal range[149]. Furthermore, bleeding times can be prolonged in patients with severe anemia of any etiology[149,150].

Because increasing the hematocrit does not shorten the bleeding time in all uremic patients, other factors likely impair platelet adhesion as well[141]. Abnormal ristocetin-induced platelet aggregation suggests that uremia induces a quantitative or qualitative abnormality in vWf or in its platelet receptor GP Ib–IX–V. However, the plasma concentration of vWf in patients with renal failureis either normal or elevated and qualitative vWf abnormalities have not been uniformly observed[142,151,152]. Nonetheless, uremic plasma can inhibit platelet adhesion to de-endothelialized human umbilical artery segments[142]. Uremic platelets also spread poorly on the subendothelium of rabbit blood vessels[153], an abnormality attributed to impaired vWf binding to activated GPIIb/IIIa, suggesting that platelet activation may be defective in uremia.

Additional observations suggest that a platelet activation defect is present in uremia. For example, uremia, and uremic plasma, can reduce agonist-stimulated fibrinogen binding to GPIIb/IIIa, platelet aggregation, and platelet secretion[154]. Platelet procoagulant activity is consistently reduced in uremia[155]. Agonist-induced biochemical

Cambridge Books Online © Cambridge University Press, 2009

responses, including a rise in cytoplasmic free calcium levels[156], release of arachidonic acid from platelet phospholipids[157], and conversion of arachidonic acid to prostaglandin endoperoxides and thromboxane A_2 can be reduced in platelets from uremic patients[158–160]. A decrease in the dense-granule content of ADP and serotonin has been observed in uremia[161], as has an increase in the concentration of cAMP[162].

Both dialysable and non-dialysable substances may be responsible for the platelet activation defect in uremia. These include small molecules such as guanidinosuccinic acid, phenolic acids, and a poorly characterized 'middle molecules'[163]. In uremic rats, prolonged bleeding times were normalized by an inhibitor of nitric oxide formation[164], suggesting that nitric oxide may be partly responsible for the defective function of uremic platelets[165]. Impaired platelet aggregation may improve following dialysis[166,167], but venous and arterial segments from uremic patients produce more prostacyclin than their normal counterparts and this is not corrected by dialysis[168,169].

When bleeding complicates a surgical procedure in a patient with renal failure or occurs spontaneously and cannot be attributed to an etiology other than uremia, there are several therapeutic maneuvers that can undertaken (Table 45.3). These manoeuvres are not uniformly effective and the choice of therapy should be based on considerations such as the severity of the bleeding, the predicted duration of therapeutic effect, and the risks of therapy.

Table 45.3. Treatment of platelet dysfunction in patients with uremia

Dialysis
Desmopressin
Red cell transfusion
Conjugated estrogens
Cryoprecipitate

Dialysis

Intensive dialysis can correct the bleeding time and bleeding diathesis in many patients, but is only partially effective in others[167,170]. Peritoneal dialysis and hemodialysis are equally effective[171,172]. If a patient undergoing dialysis bleeds, it may be worthwhile to increase the intensity of the dialysis.

Desmopressin (1-desamino-8-D-arginine vasopressin, DDAVP)

This vasopressin analogue has been reported to shorten the bleeding time in 50 to 75 per cent of patients with uremia. In many cases, surgery has been carried out safely after administration of this drug, although no controlled trial has been performed[173]. DDAVP is usually administered intravenously in a dose of 0.3 μg/kg over 15 to 30 min (maximum dose 20 μg), but it is also effective at this dose when given subcutaneously[173] or intranasally[174]. Improvement in the bleeding time is seen within 30 to 60 min of administration, lasts for approximately 4 h, and roughly correlates with the rise in the plasma levels of vWf and the appearance in the circulation of high molecular weight vWf multimers[175]. In some patients, the drug has been given repeatedly at 12– to 24-h intervals, although tachyphylaxis can occur[176].

Side effects of DDAVP administration are uncommon and generally mild. A 10 to 15% decrease in mean arterial pressure, a 20 to 30% increase in pulse rate, facial flushing, water retention, and hyponatremia leading to seizures can occur[173,177]. The latter has not been observed in patients whose kidneys cannot respond to the hormone. Rarely, stroke and myocardial infarction have been reported in uremic and non-uremic individuals with atherosclerosis after DDAVP administration[178–180]. If dialysis is not effective, DDAVP is the treatment of choice for uremic bleeding, particularly if only a short-term effect is required[175].

Red cell transfusion

Increasing the hematocrit to 27–32%, either by red blood cell transfusion or treatment with erythropoietin, is associated with correction of the bleeding time in uremic individuals[140,145–148]. The beneficial effects of red cells and DDAVP may be additive[181]. Correction of the bleeding time by increasing the red cell mass would be expected to be more durable than correction with DDAVP. A number of reports suggest that erythropoietin has an effect on platelets that is independent of an increase in hematocrit[144], perhaps by increasing the number of young platelets in the circulation[182].

Conjugated estrogens

Conjugated estrogens, at dose of 0.6 mg/kg intravenously for 5 days, have been reported to shorten the bleeding time in most uremic individuals[151,183–185] and appear to be useful in uremic patients who bleed from gastrointestinal telangiectasia[186]. Shortening of the bleeding time may be seen within 72 hrs, but the maximal effect occurs within 5 to 7 days and can persist for up to 14 days.

Cryoprecipitate

Infusion of cryoprecipitate has been reported to correct the bleeding time and to ameliorate bleeding[187,188], but

Cambridge Books Online © Cambridge University Press, 2009

others have reported inconsistent results[189]. Hemostasis may be promoted by either vWf or the platelet microparticles found within cryoprecipitate preparations[190].

Anti-platelet antibodies

Antibody binding to platelets occurs in several pathologic conditions, such as idiopathic thrombocytopenic purpura (ITP), system lupus erythematosus (SLE), and platelet alloimmunization, usually resulting in platelet destruction. In most instances, surviving platelets function normally, but in some cases of ITP, bleeding times may be shorter than expected for the degree of thrombocytopenia[191]. Conversely, some individuals with circulating antiplatelet antibodies have impaired platelet function.

The mechanism by which auto- or alloantibodies inhibit platelet function is usually not apparent, although antibody binding to specific platelet membrane proteins has been shown to be responsible in several cases. Most autoantibodies are directed against the GPIIb/IIIa complex, but antibodies directed against GPIb–IX–V, GPIa/IIa, and GPIV have been detected as well[192]. Thus, the platelets of some patients with antiplatelet antibodies may exhibit impaired platelet aggregation to ADP, epinephrine, or collagen[193–196]. Two IgG autoantibodies against GPIb have been reported that selectively inhibited ristocetin-induced platelet aggregation[197,198]. Impaired collagen-induced platelet aggregation has been associated with autoantibodies against a platelet collagen receptor, GPIa/IIa[199,200]. Antibodies have also been observed to activate platelets and induce aggregation and secretion via immune complex binding to the platelet Fc receptors, by depositing sublytic quantities of the complement C5b–9 complex on the platelet surface[201], and by binding to a specific membrane antigen[202]. This may explain the acquired storage pool disease occasionally seen in ITP or SLE[203,204].

Platelet dysfunction should be suspected in a patient with ITP or SLE who has mucocutaneous bleeding with a platelet count that is not ordinarily associated with bleeding. In these cases, the bleeding time may be longer than expected for the platelet count[203,205]. Patients (usually women) have also been reported with ‘easy bruising’ a normal platelet count, circulating antiplatelet antibodies and megathrombocytes[206]. These patients may have ITP with ‘compensated thrombocytolysis’.

Cardiopulmonary bypass

Thrombocytopenia and platelet function defects are consistent consequences of circulating blood through an extracorporeal bypass circuit[207,208]. Platelet counts typically decrease to 50% of presurgical levels and can persist for as long as several days[207,209,210]. The thrombocytopenia can be attributed to hemodilution from priming the pump with colloid or crystalloid solutions[209–211], but it also can result from platelet adhesion to artificial surfaces in the circuit, as shown in scanning electron micrographs[212–214]. Other factors include disseminated intravascular coagulation, sequestration of damaged platelets in the liver, and heparin-induced thrombocytopenia[215].

Qualitative platelet defects manifest as prolonged bleeding times, abnormal ex vivo platelet aggregation, decreased ristocetin-induced platelet agglutination, α and dense granules deficiency, and generation of platelet microparticles[207,209,210,214,216–218]. Their severity correlates with the duration of extracorporeal bypass and they generally resolve within 2 to 24 hours[219]. Bypass-induced platelet function defects likely result from platelet activation and fragmentation[218,220] due to hypothermia, contact with fibrinogen-coated synthetic surfaces, contact with the blood–air interface, cardiotomy suction, and exposure to traces of thrombin, plasmin, ADP or complement[214,221–224]. Drugs such as heparin, protamine and aspirin, as well as fibrin degradation products, can also impair platelet function[208,225,226].

The most important determinant of blood loss following cardiopulmonary surgery is the surgical procedure itself. If excessive non-surgical postoperative bleeding occurs, one should verify that the patient is no longer hypothermic and that heparin has been fully reversed. If neither of these is a factor, then administration of pharmacologic agents, along with judicious transfusions of platelets, cryoprecipitate, fresh frozen plasma and red blood cells, is appropriate. Several pharmacologic agents have been tried to assist in the management of postoperative bleeding. Although patients with excessive post-operative blood loss can respond to DDAVP, as evidenced by a shortening of the bleeding time, the results of trials using DDAVP have been contradictory, some studies showing a reduced blood loss and others showing no benefit[227,228]. Because platelet activation during bypass could be a major cause of postoperative platelet dysfunction, infusions of PGE_1, prostacyclin, or stable prostacyclin analogues have been tried in animal and humans to increase platelet cyclic AMP and reduce platelet responsiveness. Randomized trials using prostacyclin and its analogue, Iloprost, did not show a clear overall benefit[77,80]. The protease inhibitor aprotinin can reduce mediastinal blood loss and transfusion requirements[229,230]. Some studies suggest that aprotinin exerts a protective effect on platelets[229,231], but others do not[232,233]. Aprotinin does inhibit hyperfibrinolysis and this may be its sole beneficial activity at low dosage[232,234]. Other anti-

Cambridge Books Online © Cambridge University Press, 2009

fibrinolytic agents that may have a role in minimizing post-operative blood loss include ε-aminocaproic acid and tranexamic acid[235,236].

Miscellaneous disorders

Chronic liver disease has been reported to cause a prolonged bleeding time and reduced platelet aggregation and procoagulant activity[237,238]. The prolonged bleeding time in such patients may respond to infusion of DDAVP[239]. A number of defects in platelet function have been reported in patients with cirrhosis including dense granule deficiency[240], increases in the platelet content of cAMP and cGMP[241], degradation of von Willebrand factor with loss of high molecular weight multimers[242], and paradoxically, platelet hypersensitivity to botrocetin[243]. However, these defects are not specific for liver disease and the basis for the hemorrhagic diathesis associated with liver disease is multifactorial[244,245]. Patients with disseminated intravascular coagulation (DIC) can exhibit reduced platelet aggregation and acquired storage pool deficiency[246,247]. These result from platelet activation in vivo by thrombin or other agonists. In addition, elevated levels of fibrin(ogen) degradation products and low fibrinogen levels contribute to the platelet defect. However, it is difficult to assess the significance of platelet dysfunction in most patients with DIC due to the simultaneous presence of thrombocytopenia and other hemostatic defects. Platelet function abnormalities have been described in Bartter syndrome, possibly due to an inhibitory prostaglandin[248]. There are isolated reports of a slight prolongation of the bleeding time and/or ex vivo platelet function defects in a number of other conditions, including nonthrombocytopenic purpura with eosinophilia[249,250], atopic asthma and hay fever[251], acute respiratory failure[252], and Wilms tumour elaborating hyaluronic acid[253].

Hematologic disorders associated with abnormal platelet function

Chronic myeloproliferative disorders

Several factors contribute to abnormal hemostasis in the chronic myeloproliferative disorders essential thrombocythemia, polycythemia rubra vera, myelofibrosis with myeloid metaplasia, and chronic myelogenous leukemia[254]. These include the increased whole-blood viscosity characteristic of polycythemia vera[255,256], intrinsic defects in platelet function that generally do not prolong the bleeding time[257], and thrombocytosis[258–262].

Table 45.4. Biochemical abnormalities reported in platelets of patients with myeloproliferative disorders

Decreased arachidonic acid release from membrane phospholipids
Reduced cyclooxygenase and/or lipoxygenase activity
Reduced responsiveness to thromboxane A_2
Dense- and/or α-granule deficiency
Decreased membrane proteins
$\alpha 2\beta 1$ (GPIa/IIa)
α_2-adrenergic receptors
GPIb–IX–V
PGD_2 receptors
Thrombopoietin receptors
Increased membrane proteins
Platelet Fc receptors
GPIV
Reduced platelet procoagulant activity
Acquired von Willebrand disease

Bleeding occurs in about one-third of patients with myeloproliferative disorders and contributes to mortality in 10 percent. Thrombosis also occurs in one-third of cases, contributing to mortality in 15 to 40 percent[263]. Bleeding usually involves the skin or mucous membranes, but may also occur after surgery or trauma. Thrombosis can involve arteries or veins and may occur in unusual locations such as the hepatic, portal, and mesenteric circulations[263–265]. It has been difficult to predict the risk of bleeding or thrombosis in an asymptomatic patient[266], but an increased number of reticulated platelets in patients with thrombocytosis, thought to reflect an increase in platelet turnover, has been associated with an increased risk for thrombosis[267]. Vascular complications are also more likely to occur in patients older than 60 and in patients with other risk factors for vascular disease[268].

A number of functional and biochemical abnormalities have been described in the platelets of patients with myeloproliferative disorders[257] (Table 45.4). These include decreased release of arachidonic acid from membrane phospholipids[269,270], reduced conversion of arachidonic acid to prostaglandin endoperoxides or lipoxygenase products[271], reduced responsiveness to thromboxane A_2 [272,273], deficiency of dense- or α-granules[274,275], unresponsiveness to collagen due to GPIa/IIa deficiency[276], and decreased numbers of α_2-adrenergic receptors[277,278]. The latter results in a defect in epinephrine-induced aggregation, often including absence of the primary wave of aggregation, which is unusual in other conditions. In

Cambridge Books Online © Cambridge University Press, 2009

addition, reduced platelet procoagulant activity has been reported in some patients[279], as have specific platelet membrane abnormalities, including decreased amounts of GPIb–IX–V[280], decreased numbers of PGD_2 receptors[281], increased numbers of platelet Fc receptors[282], an increased amount of GPIV (CD36)[283–285], and decreased expression of thrombopoietin receptors[286]. An acquired form of von Willebrand disease has been reported in several individuals with chronic myelogenous leukemia and other myeloproliferative syndromes[287]. In these cases, there was a reduction in high molecular weight vWf multimers[288]; in some, the vWf abnormality was corrected transiently by infusion of DDAVP[284,289]. In others, the abnormalities were partially or completely corrected by cytoreductive therapy[287,290].

Leukemias and myelodysplastic syndromes

Although thrombocytopenia is the overwhelming cause of bleeding in patients with leukemia, abnormal platelet function has been described in acute myelogenous leukemia. Besides morphologic abnormalities, there may be platelet aggregation and secretion abnormalities, as well as decreased platelet procoagulant activity[291–293]. These defects are intrinsic to the platelet and probably relate to the fact that the megakaryocytes from which platelets are derived have originated from a leukemic stem cell. Identical platelet abnormalities may be seen in the myelodysplastic syndrome[291,294]. Reduced platelet aggregation has also been reported in children with acute lymphocytic leukemia[292]. The platelets of some patients wth hairy-cell leukemia exhibit storage pool deficiency or a defect in platelet activation[295–300]. A single case of acquired von Willebrand disease in association with hairy-cell leukemia has been reported[301].

Dysproteinemias

Platelet dysfunction is observed in approximately one-third of patients with IgA myeloma or Waldenström macroglobulinemia, 15% of patients with IgG multiple myeloma, occasionally in patients with monoclonal gammopathy of undetermined significance[302] and can be associated with a prolonged bleeding time, even in the absence of clinical bleeding. The platelet defect is caused by the monoclonal protein, perhaps because it interacts with the platelet surface to non-specifically inhibit platelet adhesion or stimulus–response coupling. In some cases, however, specific interactions of a monoclonal protein with platelets have been described[303,304]. Several patients with myeloma, benign monoclonal gammopathy, or chronic lymphocytic leukemia have been reported to have an acquired form of von Willebrand disease[305–307].

When clinically significant platelet dysfunction occurs in a patient with a dysproteinemia, cytoreductive therapy should be considered to reduce the production and plasma level of the monoclonal immunoglobulin[302]. Plasmapheresis can also control bleeding by reducing the level of the abnormal protein[308,309].

Table 45.5. Conditions associated with acquired von Willebrand disease

Condition
Autoimmune disorders
Systemic lupus erythematosis
Scleroderma
Plasma Cell dyscrasias
Multiple myeloma
Waldenstrom's macroglobulinemia
MGUS
Lymphoproliferative disorders
Non-Hodgkin's lymphoma
Chronic lymphocytic leukemia
Hairy cell leukemia
Myeloproliferative disorders
Hypothyroidism
Gastrointestinal angiodysplasia
Drugs
Valproic acid
Ciprofloxacin
Wilm's tumour

Acquired von Willebrand disease

Acquired von Willebrand disease[310] is a relatively rare disorder that has been seen in patients with a variety of conditions including autoimmune disorders such as systemic lupus[311] and scleroderma[312]; plasma cell dyscrasias such as multiple myeloma[313,314], Waldenstrom's macroglobulinemia[315] and MGUS[316]; lymphoproliferative disorders such as non-Hodgkin's lymphoma[317,318], chronic lymphocytic leukemia[319], and hairy cell leukemia[320]; myeloproliferative disorders[287]; hypothyroidism[321]; gastrointestinal angiodysplasia[322]; Wilm's tumour[323]; and ciprofloxacin[324] or valproic acid administration[325] (Table 45.5). In these disorders, antibodies againt vWf are present[313,314,326–328]. When acquired von Willebrand disease occurs in patients with cancer or hypothyroidism, it may result from the non-specific direct absorption of vWf onto tumour cells[301,323,329], or decreased vWf production[330,331]. The presence of an in vitro inhibitor of vWf may, or may not, be detectable depending on whether the antibody neutralizes Wf function or merely

Cambridge Books Online © Cambridge University Press, 2009

leads to accelerated vWF clearance[332]. Patient management includes infusions of desmopressin[314,319,328], vWf-containing factor VIII concentrates[333], or high dose intravenous immunoglobulin[334,335]. The latter has been efficacious in patients when acquired von Willebrand disease is associated with a lymphoproliferative disorder or monoclonal paraprotein and most likely acts by delaying vWf clearance via reticuloendothelial cell blockade[336–338]. Treatment of the underlying associated disease is only sometimes helpful[332].

References

1 George J, Shattil SJ. The clinical importance of acquired abnormalities of platelet function. *N Engl J Med* 1991; 324: 27–39.

2 Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. *Ann Rev Biochem* 2000; 69: 145–182.

3 Weiss HJ, Aledort LM. Impaired platelet/connective tissue reaction in man after aspirin ingestion. *Lancet* 1967; 2: 495–497.

4 O'Brien JR. Effect of salicylates on human platelets. *Lancet* 1968; 1: 779–783.

5 Patrono C. Aspirin as an antiplatelet drug. *N Engl J Med* 1994; 330: 1287–1294.

6 Kyrle PA, Eichler HG, Jager U, Lechner K. Inhibition of prostacyclin and thromboxane A_2 generation by low-dose aspirin at the site of plug formation in man in vivo. *Circulation* 1987; 75: 1025–1029.

7 Clarke RJ, Mayo G, Price P, FitzGerald GA. Suppression of thromboxane A_2 but not systemic prostacyclin by controlled-release aspirin. *N Engl J Med* 1991; 325: 1137–1141.

8 Jaffe EQ, Weksler BB. Recovery of endothelial cell prostacyclin production after inhibition by low doses of aspirin. *J Clin Invest* 1979; 63: 532–535.

9 Kallmann R, Nieuwenhuis HK, de Groot PG, van Gijn J, Sixma JJ. Effects of low doses of aspirin, 10 mg and 30 mg daily, on bleeding time, thromboxane production and 6-keto-PGF1α excretion in healthy subjects. *Thromb Res* 1987; 45: 355–361.

10 Nakajima H, Takami H, Yamagata K, Kariya K, Tamai Y, Nara H. Aspirin effects on colonic mucosal bleeding. *Dis Colon Rectum* 1997; 40: 1484–1488.

11 Hirsh J, Salzman EW, Harker L et al. Aspirin and other platelet active drugs. Relationship among dose, effectiveness, and side effects. *Chest* 1989; 95: 12S-18S.

12 Steering Committee of the Physicans' Health Study Research Group. Final report on the aspirin component of the ongoing physicians' health study. *N Engl J Med* 1989; 321: 129–135.

13 Davenport HW. Salicylate damage to the gastric mucosal barrier. *N Engl J Med* 1967; 276: 1307–1312.

14 Leonards JR, Levy G. The role of dosage form in aspirin-induced gastrointestinal bleeding. *Clin Pharmacol Ther* 1969; 8: 400–408.

15 Stuart MJ, Gross SJ, Elrad H, Graeber JE. Effects of acetylsalicylic acid ingestion on maternal and neonatal hemostasis. *N Engl J Med* 1982; 307: 909–912.

16 Ferraris VA, Ferraris S, Lough FC, Berry WR. Preoperative aspirin ingestion increases operative blood loss after coronary artery bypass grafting. *Ann Thorac Surg* 1988; 45: 71–74.

17 Sethi GK, Copeland JG, Goldman S, Moritz T, Zadina K, Henderson WG. Implications of preoperative administration of aspirin in patients undergoing coronary artery bypass grafting. Department of Veterans Affairs cooperative study of antiplatelet therapy. *J Am Coll Cardiol* 1990; 15: 15–20.

18 Kitchen L, Erichson RB, Sideropoulos H. Effect of drug-induced platelet dysfunction on surgical bleeding. *Am J Surg* 1982; 143: 215–217.

19 Horlocker TT, Wedel DJ, Offord KP. Does preoperative antiplatelet therapy increase the risk of hemorrhagic complications associated with regional anesthesia? *Anesth Analg* 1990; 70: 631–634.

20 Livio M, Benigni A, Vigano G, Mecca G, Remuzzi G. Moderate doses of aspirin and risk of bleeding in renal failure. *Lancet* 1986: 414–416.

21 Gaspari F, Vigano G, Orisio S, Bonati M, Livio M, Remuzzi G. Aspirin prolongs bleeding time in uremia by a mechanism distinct from platelet cyclooxygenase inhibition. *J Clin Invest* 1987; 79: 1788–1797.

22 Chesebro JH, Fuster V, Elveback LR et al. Trial of combined warfarin plus dipyridamole or aspirin therapy in prosthetic heart valve replacement: Danger of aspirin compared to dipyridamole. *Am J Cardiol* 1983; 51: 1537–1541.

23 Deykin D, Janson P, McMahon L. Ethanol potentiation of aspirin-induced prolongation of the bleeding time. *N Engl J Med* 1982; 306: 852–854.

24 James MJ, Walsh JA. Effects of aspirin and alcohol on platelet thromboxane synthesis and vascular prostacyclin synthesis. *Thromb Res* 1985; 39: 587–593.

25 Kobrinsky NL, Israels ED, Gerrard JM et al. Shortening of bleeding time by 1-deamino-8-arginine vasopressin in various bleeding disorders. *Lancet* 1984; 1: 1145–1148.

26 Lethagen S, Rugarn P. The effect of DDAVP and placebo on platelet function and prolonged bleeding time induced by oral acetyl salicylic acid intake in healthy volunteers. *Thromb Haemost* 1992; 67: 185–186.

27 Buchanan GR, Martin V, Levine PH, Scoon K, Handin RI. The effects of 'anti-platelet' drugs on bleeding time and platelet aggregation in normal human subjects. *Am J Clin Pathol* 1977; 68: 355–359.

28 Nadell J, Bruno J, Varady J, Segre EJ. Effect of naproxen and of aspirin on bleeding time and platelet aggregation. *J Clin Pharmacol* 1974; 14: 176–182.

29 Mielke CH, Jr., Kahn SB, Muschek LL, Tighe JJ, Ng KT, Minn FL. Effects of zomepirac on hemostasis in healthy adults and on platelet function in vitro. *J Clin Pharmacol* 1980; 20: 409–417.

Cambridge Books Online © Cambridge University Press, 2009

30 Thomas P, Hepburn B, Kim HC, Saidi P. Nonsteroidal anti-inflammatory drugs in the treatment of hemophilic arthropathy. *Am J Hematol* 1982; 12: 131–137.
31 McIntyre BA, Philip RB, Inwood JJ. Effect of ibuprofen on platelet function in normal subjects and hemophiliac patients. *Clin Pharmacol Ther* 1978; 24: 616–621.
32 Kasper CK, Rapaport SI. Bleeding times and platelet aggregation after analgesics in hemophilia. *Ann Intern Med* 1972; 77: 189–193.
33 Mielke CH, Jr. Comparative effects of aspirin and acetominophen on hemostasis. *Arch Intern Med* 1981; 141: 305–310.
34 Sattler FR, Weitekamp MR, Ballard JO. Potential for bleeding with the new beta-lactam antibiotics. *Ann Intern Med* 1986; 105: 924–931.
35 Pillgram-Larsen J, Wisloff F, Jorgensen JJ, Godal HC, Semb G. Effect of high-dose ampicillin and cloxacillin on bleeding time and bleeding in open-heart surgery. *Scand J Thorac Cardiovasc Surg* 1985; 19: 45–48.
36 Fass RJ, Copelan EA, Brandt JT, Moeschberger ML, Ashton JJ. Platelet-mediated bleeding caused by broad-spectrum penicillins. *J Infect Dis* 1987; 155: 1242–1248.
37 Cazenave JP, Packman MA, Guccione MA, Mustard JF. Effects of penicillin G on platelet aggregation, release and adherence to collagen. *Proc Soc Exp Biol Med* 1973; 142: 159–166.
38 Shattil SJ, Bennett JS, McDonough M, Turnbull J. Carbenicillin and penicillin G inhibit platelet function in vitro by impairing the interaction of agonists with the platelet surface. *J Clin Invest* 1980; 65: 329–337.
39 Henry D, Audet P, Shattil SJ. Relationships between the structure of penicillins and their anti-platelet activity. *Blood* 1979; 54(Suppl 1): 243a.
40 Fletcher C, Pearson C, Choi SC, Duma RJ, Evans HJ, Qureshi GD. In vitro comparison of antiplatelet effects of β-lactam penicillins. *J Lab Clin Med* 1986; 108: 217–223.
41 Sattler FR, Weitekamp MR, Sayegh A, Ballard JO. Impaired hemostasis caused by beta-lactam antibiotics. *Am J Surg* 1988; 155: 30–39.
42 Andrassy K, Koderisch J, Trenk D, Jahnchen E, Iwand A. Hemostasis in patients with normal and inpaired renal function under treatment with cefodizime. *Infection* 1987; 15: 348–350.
43 Brown RB, Klar J, Lemeshow S, Teres D, Pastides H, Sands M. Enhanced bleeding with cefoxitin or moxalactam: statistical analysis within a defined population of 1,493 pateints. *Arch Intern Med* 1986; 146: 2159–2164.
44 Bang NU, Tessler SS, Heidenreich RO, Marks CA, Mattler LE. Effects of moxalactam on blood coagulation and platelet function. *Rev Infect Dis* 1982; 4(suppl): S546–S554.
45 Rossi EC, Levin NW. Inhibition of primry ADP-induced platelet aggregation in normal subjects after the administration of nitrofurantoin (Furadantin). *J Clin Invest* 1973; 52: 2457–2467.
46 Ishikawa S, Manabe S, Wada O. Miconazole inhibition of platelet aggregation by inhibiting cyclooxygenase. *Biochem Pharmacol* 1986; 35: 1787–1792.
47 Hechler B, Eckly A, Ohlmann P, Cazenave JP, Gachet C. The P2Y1 receptor, necessary but not sufficient to support full ADP- induced platelet aggregation, is not the target of the drug clopidogrel. *Br J Haematol* 1998; 103: 858–866.
48 Geiger J, Honig-Liedl P, Schanzenbacher P, Walter U. Ligand specificity and ticlopidine effects distinguish three human platelet ADP receptors. *Eur J Pharmacol* 1998; 351: 235–246.
49 Hollopeter G, Jantzen HM, Vincent D et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* 2001; 409: 202–207.
50 McTavish D, Faulds D, Goa KL. Ticlopidine. An updated review of its pharmacology and therapeutic use in platelet-dependent disorders. *Drugs* 1992; 40: 238–259.
51 Hass WK, Easton JD, Adams HP et al. A randomized trial comparing ticlopidine hydrochloride with aspirin for the prevention of stroke in high-risk patients. *N Engl J Med* 1989; 321: 501–507.
52 Gent M, Blakely JA, Easton JD, Ellis DJ, Hachniski VC. The Canadian American Ticlopidine Study (CATS) in thromboembolic stroke. *Lancet* 1989: 1215–1220.
53 Mataiz R, Ojeda E, Perez MDC, Jiminez S. Ticlopidine and severe aplastic anemia. *Br J Haematol* 1992; 80: 125–126.
54 Garnier G, Taillan B, Pesce A, Chichmanian R-M, Dujardin P. Ticlopidine and severe aplastic anemia. *Br J Haematol* 1992; 81: 459–460.
55 Bennett CL, Weinberg PD, Rozenberg-Ben-Dror K, Yarnold PR, Kwaan HC, Green D. Thrombotic thrombocytopenic purpura associated with ticlopidine. A review of 60 cases. *Ann Intern Med* 1998; 128: 541–544.
56 Steinhubl SR, Tan WA, Foody JM, Topol EJ. Incidence and clinical course of thrombotic thrombocytopenic purpura due to ticlopidine following coronary stenting. EPISTENT Investigators. Evaluation of Platelet IIb/IIIa Inhibitor for Stenting. *JAMA* 1999; 281: 806–810.
57 Chen DK, Kim JS, Sutton DM. Thrombotic thrombocytopenic purpura associated with ticlopidine use: a report of 3 cases and review of the literature. *Arch Intern Med* 1999; 159: 311–314.
58 CAPRIE Steering Committee. A randomized, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). *Lancet* 1996; 48: 1329–1339.
59 Bennett CL, Connors JM, Carwile JM et al. Thrombotic thrombocytopenic purpura associated with clopidogrel. *N Engl J Med* 2000; 342: 1773–1777.
60 Bennett CL, Connors JM, Moake JL. Clopidogrel and thrombotic thrombocytopenic purpura. *N Engl J Med* 2000; 343: 1193–1194.
61 EPIC Investigators. Use of a monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high risk coronary angioplasty. *N Engl J Med* 1994; 330: 956–961.
62 EPILOG Investigators. Platelet glycoprotein IIb/IIIa receptor blockade and low-dose heparin during percutaneous coronary revascularization. *N Engl J Med* 1997; 336: 1689–1696.
63 Ferguson JJ, Kereiakes DJ, Adgey J et al. Safe use of platelet GP IIb/IIIa inhibitors. *Am Heart J* 1998; 135: 577–589.

Cambridge Books Online © Cambridge University Press, 2009

64 Berkowitz SD, Sane DC, Sigmon KN et al. Occurence and clinical significance of thrombocytopenia in a population undergoing high-risk percutaneous coronary revascularization. *J Am Coll Cardiol* 1998; 32: 311–319.

65 PRISM-PLUS Investigators. Inhibition of the platelet glycoprotein IIb/IIIa receptor with tirofiban in unstable angina and non-Q-wave myocardial infarction. *N Engl J Med* 1998; 338: 1488–1497.

66 PRISM Investigators. Inhibition of platelet glycoprotein IIb/IIIa with eptifibatide in patients with acute coronary syndromes. *N Engl J Med* 1998; 339: 436–443.

67 Cannon CP, McCabe CH, Borzak S et al. Randomized trial of an oral platelet glycoprotein IIb/IIIa antagonist, sibrafiban, in patients after an acute coronary syndrome. *Circulation* 1998; 97: 340–349.

68 Sane DC, Damaraju LV, Topol EJ et al. Occurrence and clinical significance of pseudothrombocytopenia during abciximab therapy. *J Am Coll Cardiol* 2000; 36: 75–83.

69 Kereiakes DJ, Essell JH, Abbottsmith CW, Broderick TM, Runyon JP. Abciximab-associated profound thrombocytopenia: therapy with immunoglobulin and platelet transfusion. *Am J Cardiol* 1996; 78: 1161–1163.

70 Berkowitz SD, Harrington RA, Rund MM, Tcheng JE. Acute profound thrombocytopenia after c7E3 Fab (abciximab) therapy. *Circulation* 1997; 95: 809–813.

71 Gresele P, Arnout J, Deckmyn H, Vermylen J. Mechanism of the antiplatelet action of dipyridamole in whole blood: modulation of adenosine concentration and activity. *Thromb Haemost* 1986; 55: 12–18.

72 FitzGerald GA. Dipyridamole. *N Engl J Med* 1987; 316: 1247–1257.

73 Diener HC, Cunha L, Forbes C, Sivenius J, Smets P, Lowenthal A. European Stroke Prevention Study. 2. Dipyridamole and acetylsalicylic acid in the secondary prevention of stroke. *J Neurol Sci* 1996; 143: 1–13.

74 Ikeda Y. Antiplatelet therapy using cilostazol, a specific PDE3 inhibitor. *Thromb Haemost* 1999; 82: 435–438.

75 Sorkin EM, Markham A. Cilostazol. *Drugs Aging* 1999; 14: 63–71.

76 Yoshitomi Y, Kojima S, Sugi T, Yano M, Matsumoto Y, Kuramochi M. Antiplatelet treatment with cilostazol after stent implantation. *Heart* 1998; 80: 393–396.

77 Walker ID, Davidson JF, Faichney A, Wheately DJ, Davidson KG. A double-blind study of prostacyclin in cardiopulmonary bypass surgery. *Br J Haematol* 1981; 49: 415–423.

78 Huddleston CB, Wareing TH, Clanton JA, Bender HW, Jr. Amelioration of the deleterious effects of platelets activated during cardiopulmonary bypass: Comparison of a thromboxane synthetase inhibitor and a prostacylin analogue. *J Thorac Cardiovasc Surg* 1985; 89: 190–195.

79 Fisher CA, Kappa JR, Sinha AK et al. Comparison of equimolar concentratons of iloprost, prostacyclin, and prostaglandin E_1 on human platelet function. *J Lab Clin Med* 1987; 109: 184–190.

80 Fish KJ, Sarnquist FH, van Steennis C et al. A prospective, randomized study of the effects of prostacyclin on platelets and blood loss during coronary bypass operations. *J Thorac Cardiovasc Surg* 1986; 91: 436–442.

81 Loscalzo J, Welch G. Nitric oxide and its role in the cardiovascular system. *Progr Cardiovasc Dis* 1995; 38: 87–104.

82 De Candia E, De Cristofaro R, Landolfi R. Thrombin-induced platelet activation is inhibited by high- and low- molecular weight heparin. *Circulation* 1999; 99: 3308–3314.

83 Salzman EW, Rosenberg RD, Smith MH, Lindon JN, Favreau L. Effect of heparin and heparin fractions on platelet aggregation. *J Clin Invest* 1980; 65: 64–73.

84 Sobel M, McNeill PM, Carlson PL et al. Heparin inhibition of von Willebrand factor-dependent platelet function in vitro and in vivo. *J Clin Invest* 1991; 87: 1787–1793.

85 Coller BS. Platelets and thrombolytic therapy. *N Engl J Med* 1990; 322: 33–42.

86 Niewiarowski S, Senyi AF, Gillies P. Plasmin-induced platelet aggregation and platelet release reaction. *J Clin Invest* 1973; 52: 1647–1659.

87 Fitzgerald DJ, Catella F, Roy L, FitzGerald GA. Marked platelet activation in vivo after intravenous streptokinase in patients with acute myocardial infarction. *Circulation* 1988; 77: 142–150.

88 Kerins DM, Roy L, FitzGerald GA, Fitzgerald DJ. Platelet and vascular function during coronary thrombolysis with tissue-type plasminogen activator. *Circulation* 1989; 80: 1718–1725.

89 Thorsen LI, Brosstad F, Gogstad G, Sletten K, Solum NO. Competitions between fibrinogen with its degradation products for interactions with the platelet–fibrinogen receptor. *Thromb Res* 1986; 44: 611–623.

90 Adelman B, Michaelson AD, Loscalzo J, Greenberg J, Handin RI. Plasmin effect on platelet glycoprotein Ib-von Willebrand factor interactions. *Blood* 1985; 65: 32–40.

91 Stricker RB, Wong D, Shiu DT, Reyes PT, Shuman MA. Activation of plasminogen by tissue plasminogen activator on normal and thrombasthenic platelets: Effects on surface proteins and platelet aggregation. *Blood* 1986; 68: 275–280.

92 Schafer AI, Adelman B. Plasmin inhibition of platelet function and of arachidonic acid metabolism. *J Clin Invest* 1985; 75: 456–461.

93 Loscalzo J, Vaughn DE. Tissue plasminogen activator promotes platelet disaggregation in plasma. *J Clin Invest* 1987; 79: 1749–1755.

94 Winters KJ, Eisenberg PR, Jaffe AS, Santoro SA. Dependence of plasmin-mediated degradation of platelet adhesive receptors on temperature and Ca^{2+}. *Blood* 1990; 76: 1546–1557.

95 Hines R, Barash PG. Infusion of sodium nitroprusside induces platelet dysfunction in vitro. *Anesthesia* 1989; 70: 611–615.

96 Kroll MH, Schafer AI. Biochemical mechanisms of platelet activation. *Blood* 1989; 74: 1181–1195.

97 Schafer AI, Alexander RW, Handin RI. Inhibition of platelet function by organic nitrate vasodilators. *Blood* 1980; 55: 649–654.

98 Hogman M, Frostell C, Arnberg H, Hedenstierna G. Bleeding

Cambridge Books Online © Cambridge University Press, 2009

time prolongation and NO inhalation. *Lancet* 1993; 341: 1664–1665.

99 Samama CM, Diaby M, Fellahi JL et al. Inhibition of platelet aggregation by inhaled nitric oxide in patients with acute respiratory distress syndrome. *Anesthesia* 1995; 83: 56–65.

100 Gries A, Bode C, Peter K et al. Inhaled nitric oxide inhibits human platelet aggregation, P-selectin expression, and fibrinogen binding in vitro and in vivo. *Circulation* 1998; 97: 1481–1487.

101 Weksler B, Gillick M, Pink J. Effect of propranolol on platelet function. *Blood* 1977; 49: 185–196.

102 Leon R, Tiarks CY, Pechet L. Some observations on the in vivo effect of propranolol on platelet aggregation and release. *Am J Hematol* 1978; 5: 117–121.

103 Ring ME, Corrigan JJ, Jr., Fenster PE. Effects of oral diltiazem on platelet function: Alone and in combination with 'low dose' aspirin. *Thromb Res* 1986; 44: 391–400.

104 Barnathan E, Addonizio VP, Shattil SJ. Interaction of verapamil with human platelet alpha-adrenergic receptors. *Am J Physiol* 1982; 242: H19–H23.

105 Weiss HJ. The effect of clinical dextran on platelet aggregation, adhesion, and ADP release in man: in vivo and in vitro studies. *J Lab Clin Med* 1967; 69: 37–46.

106 Aberg M, Hedner U, Bergentz S-E. Effect of dextran 70 on factor VIII and platelet function in von Willebrand's disease. *Thromb Res* 1978; 12: 629–634.

107 Mishler JM, IV. Synthetic plasma volume expanders – their pharmacology, safety and clinical efficacy. *Clin Haematol* 1984; 13: 75–92.

108 Cope JT, Banks D, Mauney MC et al. Intraoperative hetastarch infusion impairs hemostasis after cardiac operations. *Ann Thorac Surg* 1997; 63: 78–82.

109 Ruttmann TG, James MF, Aronson I. In vivo investigation into the effects of haemodilution with hydroxyethyl starch (200/0.5) and normal saline on coagulation. *Br J Anaesth* 1998; 80: 612–616.

110 Roberts JS, Bratton SL. Colloid volume expanders. Problems, pitfalls and possibilities. *Drugs* 1998; 55: 621–630.

111 Svelha,C, Spankova H, Mlejnkova M. The effect of tri-cyclic antidepressive drugs on adrenaline and adenosine diphosphate induced platelet aggregation. *J Pharmacol* 1966; 18: 616–617.

112 Warlow C, Ogston D, Douglas AS. Platelet function after administration of chlorpromazine to human subjects. *Haemostasis* 1976; 5: 21–26.

113 Corbin F, Blaise G, Sauve R. Differential effect of halothane and forskolin on platelet cytosolic Ca2+ mobilization and aggregation. *Anesthesiology* 1998; 89: 401–410.

114 Heesch CM, Negus BH, Steiner M et al. Effects of in vivo cocaine administration on human platelet aggregation. *Am J Cardiol* 1996; 78: 237–239.

115 Jennings LK, White MM, Sauer CM, Mauer AM, Robertson JT. Cocaine-induced platelet defects. *Stroke* 1993; 24: 1352–1359.

116 Togna G, Graziani M, Sorrentino C, Caprino L. Prostanoid production in the presence of platelet activation in hypoxic cocaine-treated rats. *Haemostasis* 1996; 26: 311–318.

117 Ahr DJ, Scialla SJ, Kimball DB, Jr. Acquired platelet dysfunction following mithramycin therapy. *Cancer* 1978; 41: 448–454.

118 Panella TJ, Peters,W, White JG, Hannun YA, Greenberg CS. Platelets acquire a secretion defect after high-dose chemotherapy. *Cancer* 1990; 65: 1711–1716.

119 Pogliani EM, Fantasia R, Lambertenghi-Deliliers G, Cofranesco E. Daunorubicin and platelet function. *Thromb Haemost* 1981; 45: 38–42.

120 McKenna R, Ahmad T, Ts'ao C-H, Frischer H. Gluthathione reductase deficiency and platelet dysfunction induced by 1,3-bis(2-chloroethyl)-1-nitrosourea. *J Lab Clin Med* 1983; 102: 102–115.

121 Karolak L, Chandra A, Kahn W et al. High-dose chemotherapy-induced platelet defect: Inhibition of platelet signal transduction pathways. *Mol Pharmacol* 1993; 43: 37–44.

122 Vanrenterghem Y, Roels L, Lerut T et al. Thromboembolic complications and hemostatic changes in cylcosporine-treated cadaveric kidney allograft recipients. *Lancet* 1985; 1: 999–1002.

123 Cohen H, Neild GH, Patel R, Mackie IJ, Mackin SJ. Evidence for chronic platelet hyperaggregability and in vivo platelet activation in cyclosporine treated renal allograft recipients. *Thromb Res* 1988; 49: 91–101.

124 Grace AA, Barradus MA, Mikhailidis D et al. Cyclosporine A enhances platelet aggregation. *Kidney Int* 1987; 32: 889–895.

125 Thomson C, Forbes CD, Prentice CRM. A comparison of the effects of antihistamines on platelet function. *Thromb Diath Haemorrh* 1973; 30: 547–556.

126 Group TPT. Platelet function during long term treatment with ketanserin of claudicating patients with peripheral atherosclerosis: a multicenter, double-blind, placebo-controlled trial. *Thromb Res* 1989; 55: 13–23.

127 Parvez Z, Moncada R, Fareed J, Messmore HL. Antiplatelet action of intravascular contrast media. *Invest Radiol* 1984; 19: 208–211.

128 Rao AK, Rao VM, Willis J, Beckett C, Steiner RM. Inhibition of platelet function by contrast media: Iopamidol and Hexabrix are less inhibitory than Conray-60. *Radiology* 1985; 156: 311–313.

129 Goodnight SH, Harris WS, Conner WE. The effects of ω^{-3} fatty acids on platelet composition and function in man: a prospective, controlled study. *Blood* 1981; 58: 880–885.

130 Moncada S, Higgs EA. Arachidonate metabolism in blood cells and the vessel wall. *Clin Haematol* 1986; 15: 273–292.

131 Leaf A, Weber PC. Cardiovascular effects of n-3 fatty acids. *N Engl J Med* 1988; 318: 549–557.

132 Hammerschmidt DE. Szechwan purpura. *N Engl J Med* 1980; 302: 1191–1193.

133 Srivastava KC. Onion exerts antiaggregatory effects by altering arachidonic acid metabolism in platelets. *Prostaglandins Leukotrienes Med* 1986; 24: 43–50.

134 Apitz-Castro R, Ledezma E, Escalante J, Jain MK. The molecular basis of the antiplatelet action of ajoene: Direct interaction with the fibrinogen receptor. *Biochem Biophys Res Commun* 1986; 141: 145–150.

135 Apitz-Castro R, Escalante J, Vargas R, Jain MK. Ajoene, the

Cambridge Books Online © Cambridge University Press, 2009

antiplatelet principle of garlic, synergistically potentiates the antiaggregatory action of prostacylin, forskolin, indomethacin and dipyridamole on human platelets. *Thromb Res* 1986; 42: 303–311.
136 Srivastava KC. Extracts from two frequently consumed spices- cumin (*Cuminum cyminum*) and turmeric (*Curcuma longa*) inhibit platelet aggregation and alter eicosanoid biosynthesis in human blood platelets. *Prostaglandins Leuko Essent Fatty Acids* 1993; 37: 57–64.
137 Remuzzi G. Bleeding disorders in uremia: pathophysiology and treatment. *Adv Nephrol* 1989; 18: 171–186.
138 Rosenbaum R, Hoffstein PE, Stanley RJ, Klahr S. Use of computerized tomography to diagnose complications of percutaneous renal biopsy. *Kidney Int* 1978; 14: 87–92.
139 Diaz-Buxo JA, Donadio JVJ. Complications of percutaneous renal biopsy: an analysis of 1000 consecutive biopsies. *Clin Nephrol* 1975; 4: 223–227.
140 Weigert AL, Schafer AI. Uremic bleeding: pathogenesis and therapy. *Am J Med Sci* 1998; 316: 94–104.
141 Castillo R, Lozano T, Escolar G et al. Defective platelet adhesion on vessel subendothelium in uremic patients. *Blood* 1986; 68: 337–342.
142 Zwaginga JJ, Ijsseldijk MJW, Beeser-Visser N, de Groot PG, Vos J, Sixma JJ. High von Willebrand factor concentration compensates a relative adhesion defect in uremic blood. *Blood* 1990; 75: 1498–1508.
143 Zwaginga JJ, Ijsseldijk I, de Groot PG, Vos J, de Bos Kuil RL, Sixma JJ. Defects in platelet adhesion and aggregate formation in uremic bleeding disorder can be attributed to factors in plasma. *Arteriosc Thromb* 1991; 11: 733–744.
144 Tang WW, Stead RA, Goodkin DA. Effects of epoetin alfa on hemostasis in chronic renal failure. *Am J Nephrol* 1998; 18: 263–273.
145 Fernandez F, Goudable C, Sie P et al. Low haematocrit and prolonged bleeding time in uraemic patients: Effect of red cell transfusions. *Br J Haematol* 1985; 59: 139–148.
146 Livio M, E. G, Marchesi D, Mecca G, Remuzzi G. Uraemic bleeding: Role of anaemia and beneficial effect of red cell transfusions. *Lancet* 1982; 2: 1013–1015.
147 Moia M, Mannucci PM, Vizzotto L, Casati S, Cattaneo M, Ponticelli C. Improvement in the haemostatic defect of uraemia after treatment with recombinant human erythropoietin. *Lancet* 1987; 2: 1227–1229.
148 Vigano G, Benigni A, Mendogni D, Mingardi G, Mecca G, Remuzzi G. Recombinant human erythropoietin to correct uremic bleeding. *Am J Kid Dis* 1991; 18: 44–49.
149 Small M, Lowe GDO, Cameron E, Forbes CD. Contribution to the hematocrit to the bleeding time. *Haemostasis* 1983; 13: 379–384.
150 Anonymous. The bleeding time and the haematocrit. *Lancet* 1984; 1: 997–998.
151 Livio M, Mannucci PM, Vigano G et al. Conjugated estrogens for the management of bleeding associated with renal failure. *N Engl J Med* 1986; 315: 731–735.
152 Sloand EM, Sloand JA, Prodouz K et al. Reduction of platelet glycoprotein Ib in uremia. *Br J Haematol* 1991; 77: 375–381.
153 Escolar G, Cases A, Bastida E et al. Uremic platelets have a functional defect affecting the interaction of von Willebrand factor with glycoprotein IIb–IIIa. *Blood* 1990; 76: 1336–1340.
154 Di Minno G, Cerbone A, Usberti M et al. Platelet dysfunction in uremia. II. Correction by arachidonic acid of the impaired exposure of fibrinogen receptors by adenosine diphosphate or collagen. *J Lab Clin Med* 1986; 108: 246–252.
155 Rabiner SF, Hrodek O. Platelet factor 3 in normal subjects and patients with renal failure. *J Clin Invest* 1968; 47: 901–912.
156 Ware JA, Clark BA, Smith M, Salzman EW. Abnormalities of cytoplasmic Ca^{2+} in platelets from patients with uremia. *Blood* 1989; 73: 172–176.
157 Rao AK. Uraemic platelets. *Lancet* 1986; 1: 913.
158 Remuzzi G, Benigni A, Dodesini P et al. Reduced platelet thromboxane formation in uremia: Evidence for a functional cyclooxgenase defect. *J Clin Invest* 1983; 71: 762–768.
159 Winter M, Frampton G, Bennett A, Machin SJ, Cameron JS. Synthesis of thromboxane B_2 in uraemia and the effects of dialysis. *Thromb Res* 1983; 30: 265–272.
160 Bloom A, Greaves M, Preston FE, Brown CB. Evidence against a platelet cyclooxygenase defect in uraemic subjects on chronic haemodialysis. *Br J Haematol* 1986; 62: 143–149.
161 Eknoyan G, Brown CH. Biochemical abnormalities of platelets in renal failure. Evidence for decreased platelet serotonin, adenosine diphosphate and Mg-dependent adenosine triphosphatase. *Am J Nephrol* 1981; 1: 17–23.
162 Vlachoyannis J, Schoeppe W. Adenylate cyclase activity and cAMP content of human platelets in uraemia. *Eur J Clin Invest* 1982; 12: 379–381.
163 Bazilinski N, Shaykh M, Dunea G et al. Inhibition of platelet function by uremic middle molecules. *Nephron* 1985; 40: 423–428.
164 Remuzzi G, Perico N, Zoja C, Corna D, Macconi D, Vigano G. Role of endothelium-derived nitric oxide in the bleeding tendency of uremia. *J Clin Invest* 1990; 86: 1768–1771.
165 Aiello S, Noris M, Todeschini M et al. Renal and systemic nitric oxide synthesis in rats with renal mass reduction. *Kidney Int* 1997; 52: 171–181.
166 Remuzzi G, Livio M, Marchiaro G, Mecca G, de Gaetano G. Bleeding in renal failure: Altered platelet function in chronic uraemia only partially corrected by haemodialysis. *Nephron* 1978; 22: 347–353.
167 Castaldi PA, Sydney MB. The bleeding disorder of uraemia. *Lancet* 1966; 2: 66–69.
168 Livio M, Benigni A, Remuzzi G. Coagulation abnormalities in uremia. *Semin Nephrol* 1985; 5: 82–90.
169 Remuzzi G, Cavenaghi AE, Mecca G, Donati MD, de Gaetano G. Prostacyclin-like activity and bleeding in renal failure. *Lancet* 1977; 2: 1195–1197.
170 Hutton RA, O'Shea MJ. Haemostatic mechanism in uraemia. *J Clin Pathol* 1968; 21: 406.
171 Stewart JH, Castaldi PA. Uraemic bleeding: A reversible platelet defect corrected by dialysis. *Q J Med, New Series XXXVI* 1967; 143: 409–411.
172 Lindsay RM, Friesen M, Koens F, Linton AL, Oreopoulos D, de

Cambridge Books Online © Cambridge University Press, 2009

Verber G. Platelet function in patients on long-term peritoneal dialysis. *Clin Nephrol* 1976; 6: 335–339.

173 Mannucci PM. Desmopressin: a non-transfusional form of treatment for congenital and acquired bleeding disorders. *Blood* 1988; 72: 1449–1455.

174 Rose EH, Aledort LM. Nasal spray desmopressin (DDAVP) for mild hemophilia A and von Willebrand disease. *Ann Int Med* 1991; 114: 563–568.

175 Mannucci PM. Desmopressin (DDAVP) for treatment of disorders of hemostasis. *Prog Hemost Thromb* 1986; 8: 19–45.

176 Canavese C, Salomone M, Pacitti A et al. Reduced response of uraemic bleeding time to repeated doses of desmopressin. *Lancet* 1985; 1: 867–868.

177 Bichet DG, Razi M, Lonegran M, Arthur M-F. 1–Desamino [8-D-arginine] vasopressin (dDAVP) decreases blood pressure and increases pulse rate in normal individuals. *Thromb Haemost* 1988; 60: 348–349.

178 Byrnes JJ, Larcada A, Moake JL. Thrombosis following desmopressin for uremic bleeding. *Am J Hematol* 1988; 28: 63–65.

179 Anonymous. Desmopressin and arterial thrombosis. *Lancet* 1989; 1: 938–939.

180 Mannucci PM. Desmopressin and thrombosis. *Lancet* 1989; 2: 675–676.

181 Gotti E, Mecca G, Valentino C, Cortinovis E, Bertani T, Remuzzi G. Renal biopsy in patients with acute renal failure and prolonged bleeding time. *Lancet* 1984; 2: 978–979.

182 Tassies D, Reventer JC, Cases A, Calls J, Escolar G, Ordinas A. Effect of recombinant human erythropoietin treatment on circulating reticulated platelets in uremic patients: association with early improvement in platelet function. *Am J Hematol* 1998; 59: 105–109.

183 Liu YK, Kosfeld RE, Marcum SG. Treatment of uremic bleeding with conjugated estrogen. *Lancet* 1984; 2: 887–890.

184 Vigano G, Gaspari F, Locatelli M, Pusineri F, Bonati M, Remuzzi G. Dose-effect and pharmacokinetics of estrogens given to correct bleeding time in uremia. *Kidney Int* 1988; 34: 853–858.

185 Heistinger M, Stockenhuber F, Schneider B et al. Effect of conjugated estrogens on platelet function and prostacyclin generation in CRF. *Kidney Int* 1990; 38: 1181–1186.

186 Bronner MH, Pate MD, Cunningham JT. Estrogen-progesterone therapy for bleeding of gastrointestinal telangiectasias in chronic renal failure. *Ann Intern Med* 1986; 105: 371–374.

187 Juhl A. DDAVP, cryoprecipitate and highly 'purified' factor VIII concentrate in uremia. *Nephron* 1986; 43: 305–306.

188 Janson PA, Jubelirer SJ, Weinstein MS, Deykin D. Treatment of bleeding tendency in uremia with cryoprecipitate. *N Engl J Med* 1980; 303: 1318–1322.

189 Triulzi DJ, Blumber N. Variability in response to cryoprecipitate treatment for hemostatic defects in uremia. *Yale J Biol Med* 1990; 63: 1–7.

190 George JN. Platelet membrane microparticles in blood bank fresh frozen plasma and cryoprecipitate. *Blood* 1986; 68: 307–309.

191 Thompson AR, Harker LA. Approach to bleeding disorders. *Manual of Hemostasis and Thrombosis*. Philadelphia: F.A. Davis, 1983: 57–64.

192 George JN, El-Harake MA, Raskob GE. Chronic idiopathic thrombocytopenic purpura. *N Engl J Med* 1994; 331: 1207–1215.

193 Clancy R, Jenkins E, Firkin B. Qualitative platelet abnormalities in idopathic thrombocytopenic purpura. *N Engl J Med* 1972; 286: 622.

194 Heyns DA, Fraser J, Retief FP. Platelet aggregation in chronic idiopathic thrombocytopenic purpura. *J Clin Pathol* 1978; 31: 1239–1243.

195 Regan MG, Lackner H, Karpatkin S. Platelet function and coagulation profile in lupus erythematosus. *Am J Med* 1974; 81: 462–468.

196 Dorsch CA, Meyerhoff J. Mechanisms of abnormal platelet aggregation in systemic lupus erythematosus. *Arthritis Rheum* 1982; 25: 966–973.

197 Stricker RB, Wong D, Saks SR, Corash L, Shuman MA. Acquired Bernard-Soulier syndrome: Evidence for the role of a 210,000-molecular weight protein in the interaction of platelets with von Willebrand factor. *J Clin Invest* 1985; 76: 1274–1278.

198 Devine DV, Currie MS, Rosse WF, Greenberg CS. Pseudo-Bernard–Soulier syndrome: Thrombocytopenia caused by autoantibody to platelet glycoprotein Ib. *Blood* 1987; 70: 428–431.

199 Deckmyn H, Zhang J, Van Houtte E, Vermylen J. Production and nucleotide sequence of an inhibitory human IgM autoantibody directed against platelet glycoprotein Ia/IIa. *Blood* 1994; 84: 1968–1974.

200 Dromigny A, Triadou P, Lesavre P, Morel-Kopp MC, Kaplan C. Lack of platelet response to collagen associated with autoantibodies against glycoprotein (GP) Ia/IIa and Ib/IX leading to the discovery of SLE. *Hematol Cell Ther* 1996; 38: 355–357.

201 Wiedmer T, Ando B, Sims PJ. Complement C5b-9-stimulated platelet secretion is associated with a calcium-initiated activation of cellular protein kinases. *J Biol Chem* 1987; 262: 13674–13681.

202 Sugiyama T, Okuma M, Ushikubi F, Sensaki S, Kanaji K, Uchino H. A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia. *Blood* 1987; 69: 1712–1720.

203 Weiss HJ, Rosove MH, Lages BA, Kaplan KL. Acquired storage pool deficiency with increased platelet-associated IgG. *Am J Med* 1980; 69: 711–717.

204 Meyerhoff J, Dorsch CA. Decreased platelet serotonin levels in systemic lupus erythematosus. *Arthritis Rheum* 1981; 24: 1495–1500.

205 Stuart MJ, Kelton JG, Allen JB. Abnormal platelet function and arachidonate metabolism in chronic idiopathic thrombocytopenic purpura. *Blood* 1981; 58: 326–329.

206 Lackner H, Karpatkin S. On the 'easy bruising' syndrome with normal platelet count: a study of 75 patients. *Ann Intern Med* 1975; 83: 190–196.

207 Harker LA, Malpass TW, Branson HE, Slichter SJ. Mechanism

Cambridge Books Online © Cambridge University Press, 2009

of abnormal bleeding in patients undergoing cardiopulmonary bypass: acquired transient platelet dysfunction associated with selective alpha-granule release. *Blood* 1980; 56: 824–834.

208 Khuri SF, Valeri CR, Loscalzo J et al. Heparin causes platelet dysfunction and induces fibrinolysis before cardiopulmonary bypass. *Ann Thorac Surg* 1995; 60: 1008–1014.

209 Mammen EF, Koets MH, Washington BC et al. Hemostasis changes during cardiopulmonary bypass surgery. *Semin Thromb Haemost* 1985; 11: 281.

210 Khuri SF, Wolfe JA, Josa M et al. Hematologic changes during and after cardiopulmonary bypass and their relationship to the bleeding time and nonsurgical blood loss. *J Thorac Cardiovasc Surg* 1992; 104: 94–107.

211 Martin JF, Daniel TD, Trowbridge EA. Acute and chronic changes in platelet volume and count after cardiopulmonary bypass induced thrombocytopenia in man. *Thromb Haemost* 1987; 57: 55–58.

212 Chandler AB, Hutson MS. Platelet plug formation in an extracorporeal unit. *Am J Clin Pathol* 1975; 64: 101–107.

213 Uniyal S, Brash JL. Patterns of adsorption of proteins from human plasma onto foreign surfaces. *Thromb Haemost* 1982; 47: 285–290.

214 Lindon JN, McManama, Kushner L. Does the conformation of adsorbed fibrinogen dictate platelet interactions with artificial surfaces? *Blood* 1986; 68: 355–362.

215 Singer RL, Mannion JD, Bauer TL, Armenti FR, Edie RN. Complications from heparin-induced thrombocytopenia in patients undergoing cardiopulmonary bypass. *Chest* 1993; 104: 1436–1440.

216 McKenna R, Bachmann F, Whittaker B et al. The hemostatic mechanism after open heart surgery. II. Frequency of abnormal platelet functions during and after extracorporeal circulation. *J Thorac Cardiovasc Surg* 1975; 70: 298–308.

217 Beurling-Harbury C, Galvan CA. Acquired decrease in platelet secretory ADP associated with increased post-operative bleeding in post-cardiopulmonary bypass patients and in patients with severe valvular heart disease. *Blood* 1978; 52: 13–23.

218 Abrams CS, Ellison N, Budzynski AZ, Shattil S. Direct detection of activated platelets and platelet-derived microparticles in humans. *Blood* 1990; 75: 128–138.

219 Woodman RC, Harker LA. Bleeding complications associated with cardiopulmonary bypass. *Blood* 1990; 76: 1680–1697.

220 George JN, Pickett EB, Saucerman S et al. Platelet surface glycoproteins. Studies on resting and activated platelets and platelet membrane microparticles in normal subjects, and observations in patients during adult respiratory distress syndrome and cardiac surgery. *J Clin Invest* 1986; 78: 340–348.

221 Bachmann F, McKenna R, Cole ER, Najafi H. The hemostatic mechanism after open heart surgery. I. Studies on plasma coagulation factors and fibrinolysis in 512 patients after extracorporeal circulation. *J Thorac Cardiovasc Surg* 1975; 70: 76–85.

222 Gluszko P, Ricinski B, Musial J et al. Fibrinogen receptors in platelet adhesion to surfaces of extracorporeal circuit. *Am J Physiol* 1987; 252: H615–H621.

223 van den Dungen JJ, Karliczek GF, Brenken U, Homan van der Heide JN, Wildevuur CR. Clinical study of blood trauma during perfusion with membrane and bubble oxygenators. *Thorac Cardiovasc Surg* 1982; 83: 108–116.

224 Ferraris VA, Ferraris SP, Singh A et al. The platelet thrombin receptor and postoperative bleeding. *Ann Thorac Surg* 1998; 65: 352–358.

225 Kestin AS, Valeri CR, Khuri SF et al. The platelet function defect of cardiopulmonary bypass. *Blood* 1993; 82: 107–117.

226 Weksler BB, Pett SB, Alonso D et al. Differential inhibition of aspirin of vascular prostaglandin synthesis in atherosclerotic patients. *N Engl J Med* 1983; 308: 800–805.

227 Hackmann T, Gascoyne R, Naiman SC et al. A trial of desmopressin to reduce blood loss in uncomplicated cardiac surgery. *N Engl J Med* 1989; 321: 1437–1444.

228 Seear MD, Wadsworth LD, Rogers PC, Sheps S, Ashmore PG. The effect of desmopressin acetate (DDAVP) on postoperative blood loss after cardiac operations in children. *J Thorac Cardiovasc Surg* 1989; 98: 217–219.

229 van Oeveren W, Harder MP, Roozendaal KJ, Eijsman L, Wildevuur CRH. Aprotinin protects platelets against the initial effect of cardiopulmonary bypass. *J Thorac Cardiovasc Surg* 1990; 99: 788–797.

230 Hardy J-F, Desroches J. Natural and synthetic antifibrinolytics in cardiac surgery. *Can J Anesthesiol* 1992; 39: 353–365.

231 Speekenbrink RG, Wildevuur CR, Sturk A, Eijsman L. Low-dose and high-dose aprotinin improve hemostasis in coronary operations. *J Thorac Cardiovasc Surg* 1996; 112: 523–530.

232 Orchard MA, Goodchild CS, Prentice CR et al. Aprotinin reduces cardiopulmonary bypass-induced blood loss and inhibits fibrinolysis without influencing platelets. *Br J Haematol* 1993; 85: 533–541.

233 Wahba A, Black G, Koksch M et al. Aprotinin has no effect on platelet activation and adhesion during cardiopulmonary bypass. *Thromb Haemost* 1996; 75: 844–848.

234 Mastroroberto P, Chello M, Zofrea S, Marchese AR. Suppressed fibrinolysis after administration of low-dose aprotinin: reduced level of plasmin-alpha2-plasmin inhibitor complexes and postoperative blood loss. *Eur J Cardiothorac Surg* 1995; 9: 143–145.

235 Pinosky ML, Kennedy DJ, Fishman RL et al. Tranexamic acid reduces bleeding after cardiopulmonary bypass when compared to epsilon aminocaproic acid and placebo. *J Card Surg* 1997; 12: 330–338.

236 Penta de Peppo A, Pierri MD, Scafuri A et al. Intraoperative antifibrinolysis and blood-saving techniques in cardiac surgery. Prospective trial of 3 antifibrinolytic drugs. *Tex Heart Inst J* 1995; 22: 231–6.

237 Krauss JS, Jonah MH. Platelet dysfunction (thrombocytopathy) in extra-hepatic biliary obstruction. *Southern Med J* 1982; 75: 506–507.

238 Hillbom M, Muuronen A, Neiman J. Liver disease and platelet function in alcoholics. *Br Med J* 1987; 295: 581.

Cambridge Books Online © Cambridge University Press, 2009

239 Mannucci PM, Vicente V, Vianello L et al. Controlled trial of desmopressin in liver cirrhosis and other conditions associated with a prolonged bleeding time. *Blood* 1986; 67: 1148–1153.
240 Laffi G, Marra F, Gresele P et al. Evidence for a storage pool defect in platelets from cirrhotic patients with defective aggregation. *Gastroenterology* 1992; 103: 641–646.
241 Laffi G, Marra F, Failli P et al. Defective signal transduction in platelets from cirrhotics is associated with increased cyclic nucleotides. *Gastroenterology* 1993; 105: 148–156.
242 Federici AB, Berkowitz SD, Lattuada A, Mannucci PM. Degradation of von Willebrand factor in patients with acquired clinical conditions in which there is heightened proteolysis. *Blood* 1993; 81: 720–725.
243 Beer JH, Clerici N, Baillod P, von Felten A, Schlappritzi E, Buchi L. Quantitative and qualitative analysis of platelet GPIb and von Willebrand factor in liver cirrhosis. *Thromb Haemost* 1995; 73: 601–609.
244 Stein SF, Harker LA. Kinetic and functional studies of platelets, fibrinogen, and plasminogen in patients with hepatic cirrhosis. *J Lab Clin Med* 1982; 99: 217–230.
245 Violi F, Leo R, Vezza E, Basili S, Cordova C, Balsano F. Bleeding time in patients with cirrhosis: relation with degree of liver failure and clotting abnormalities. C.A.L.C. Group. Coagulation Abnormalities in Cirrhosis Study Group. *J Hepatol* 1994; 20: 531–536.
246 Pareti FI, Capitanio A, Mannucci L. Acquired storage pool disease in platelets during disseminated intravascular coagulation. *Blood* 1976; 48: 511–515.
247 Pareti FI, Capitanio A, Mannucci L, Ponticelli C, Mannucci PM. Acquired dysfunction due to the circulation of 'exhausted' platelets. *Am J Med* 1980; 69: 235–240.
248 Stoff JS, Stemerman M, Steer M, Salzman E, Brown RS. A defect in platelet aggregation in Bartter's syndrome. *Am J Med* 1980; 68: 171–180.
249 Lim SH, Tan CE, Agasthian T, Chew LS. Acquired platelet dysfunction with eosinophilia: review of seven adult cases. *J Clin Pathol* 1989; 42: 950–952.
250 Poon MC, Ng SC, Coppes MJ. Acquired platelet dysfunction with eosinophilia in white children. *J Pediatr* 1995; 126: 959–961.
251 Szczeklik A, Milner PC, Birch J, Watkins J, Martin JF. Prolonged bleeding time, reduced platelet aggregation, altered PAF-acether sensitivity and increased platelet mass are a trait of asthma and hay fever. *Thromb Haemost* 1986; 56: 283–287.
252 Carvalho AC, Quinn DA, DeMarinis SM, Beitz JG, Zapol WM. Platelet function in acute respiratory failure. *Am J Hematol* 1987; 25: 377–388.
253 Bracey AW, Wu AH, Aceves J, Chow T, Carlile S, Hoots WK. Platelet dysfunction associated with Wilms tumor and hyaluronic acid. *Am J Hematol* 1987; 24: 247–257.
254 Landolfi R, Rocca B, Patrono C. Bleeding and thrombosis in myeloproliferative disorders: mechanisms and treatment. *Crit Rev Oncol Hematol* 1995; 20: 203–222.
255 Murphy S. Polycythemia vera. *Disease-a-Month* 1992; 38: 165–211.
256 Wasserman LR, Gilbert H. The treatment of polycythemia vera. *Med Clin N Am* 1966; 50: 1501–1518.
257 Schafer AI. Essential thrombocythemia. *Prog Hemost Thromb* 1991; 10: 69–96.
258 Mitus AJ, Barbui T, Shulman LN et al. Hemostatic complications in young patients with essential thrombocythemia. *Am J Med* 1990; 88: 371–375.
259 Lahuerta-Palacios JJ, Bornstein R, Fernandez-Debora FJ et al. Controlled and uncontrolled thrombocytosis. Its clinical role in essential thrombocytosis. *Cancer* 1988; 61: 1207–1212.
260 Kessler CM, Klein HG, Havlik RJ. Uncontrolled thrombocytosis in chronic myeloproliferative disorders. *Br J Haematol* 1982; 50: 157–167.
261 McIntyre KJ, Hoagland HC, Silverstein MN, Petitt RM. Essential thrombocythemia in young adults. *Mayo Clin Proc* 1991; 66: 149–154.
262 Bellucci S, Janvier M, Tobelem G et al. Essential thrombocythemias. Clinical evolutionary and biological data. *Cancer* 1986; 58: 2440–2447.
263 Schafer AI. Bleeding and thrombosis in the myeloproliferative disorders. *Blood* 1984; 64: 1–12.
264 Murphy S. Thrombocytosis and thrombocythaemia. *Clin Haematol* 1983; 12: 89–106.
265 Mitchell MC, Boitnott JK, Kaufman S, Cameron JL, Maddrey WC. Budd–Chiari syndrome: Etiology, diagnosis and management. *Medicine* 1982; 61: 199–218.
266 Kessler CM, Klein HG, Havlik RJ. Uncontrolled thrombocytosis in chronic myeloproliferative disorders. *Br J Haematol* 1982; 50: 157–167.
267 Rinder HM, Schuster JE, Rinder CS, Wang C, Schweidler HJ, Smith BR. Correlation of thrombosis with increased platelet turnover in thrombocytosis. *Blood* 1998; 91: 1288–1294.
268 Besses C, Cervantes F, Pereira A et al. Major vascular complications in essential thrombocythemia: a study of the predictive factors in a series of 148 patients. *Leukemia* 1999; 13: 150–154.
269 Jubilirer SJ, Russell F, Faillacourt R, Deykin D. Platelet arachidonic acid metabolism and platelet function in ten patients with chronic myelogenous leukemia. *Blood* 1980; 56: 728–731.
270 Pareti FI, Gugliotta L, Mannucci L, Guarini A, Mannucci PM. Biochemical and metabolic aspects of platelet dysfunction in chronic myeloproliferative disorders. *Thromb Haemost* 1982; 47: 84–89.
271 Schafer AI. Deficiency of platelet lipoxygenase activity in myeloproliferative disorders. *N Engl J Med* 1982; 306: 381–386.
272 Okuma M, Takayama H, Uchino H. Subnormal platelet response to thromboxane A_2 in a patient with chronic myeloid leukaemia. *Br J Haematol* 1982; 51: 469–477.
273 Ushikubi F, Okuma M, Kanaji K et al. Hemorrhagic thrombocytopathy with platelet thromboxane A_2 receptor abnormality: defective signal transduction with normal binding activity. *Thromb Haemost* 1987; 57: 158–164.
274 Malpass TW, Savage B, Hanson SR, Slichter SJ, Harker LA. Correlation between prolonged bleeding time and depletion

Cambridge Books Online © Cambridge University Press, 2009

of platelet dense granule ADP in patients with myelodysplastic and myeloproliferative disorders. *J Lab Clin Med* 1984; 103: 894–904.

275 Mohri H. Acquired von Willebrand disease and storage pool disease in chronic myelocytic leukemia. *Am J Hematol* 1986; 22: 391–401.

276 Handa M, Wantanabe K, Kawai Y et al. Platelet unresponsiveness to collagen: involvement of glycoprotein Ia-IIa ($\alpha_2\beta_1$ integrin) deficiency associated with a myeloproliferative disorder. *Thromb Haemost* 1995; 73: 521–528.

277 Kaywin P, McDonough M, Insel PA, Shattil SJ. Platelet function in essential thrombocythemia: Decreased epinephrine responsiveness associated with a deficiency of platelet alpha-adrenergic receptors. *N Engl J Med* 1978; 299: 505–509.

278 Swart SS, Pearson D, Wood JK, Barnett DB. Functional significance of the platelet alpha$_2$-adrenoceptor: Studies in patients with myeloproliferative disorders. *Thromb Res* 1984; 33: 531–541.

279 Walsh PN, Murphy S, Barry WE. The role of platelets in the pathogenesis of thrombosis and hemorrhage in patients with thrombocytosis. *Thromb Haemost* 1977; 38: 1085–1096.

280 Berndt MC, Kabral A, Grimsley P, Watson N, Robertson TI, Bradstock KF. An acquired Bernard–Soulier-like platelet defect associated with juvenile myelodysplastic syndrome. *Br J Haematol* 1988; 68: 97–101.

281 Cooper B, Schafer AI, Puchalsky D, Handin RI. Platelet resistance to prostaglandin D$_2$ in patients with myeloproliferative disorders. *Blood* 1978; 52: 618–626.

282 Moore A, Nachman RL. Platelet Fc receptor: Increased expression in myeloproliferative disease. *J Clin Invest* 1981; 67: 1064–1071.

283 Bolin RB, Okumura T, Jamieson GA. Changes in distribution of platelet membrane glycoproteins in patients with myeloproliferative disorders. *Am J Hematol* 1977; 3: 63–71.

284 Eche N, Sie P, Caranobe C, Nouvel C, Pris J, Boneu B. Platelets in myeloproliferative disorders. III. Glycoprotein profile in relation to platelet function and platelet density. *Scand J Haematol* 1981; 26: 123–129.

285 Thibert V, Bellucci S, Cristofari M, Gluckman E, Legrand C. Increased platelet CD36 constitutes a common marker in myeloproliferative disorders. *Br J Haematol* 1995; 91: 618–624.

286 Moliterno AR, Hankins D, Spivak JL. Impaired expression of the thrombopoietin receptor by platelets from patients with polycythemia vera. *N Engl J Med* 1998; 338: 572–580.

287 Budde U, Schaefer G, Mueller N et al. Acquired von Willebrand's disease in the myeloproliferative syndrome. *Blood* 1984; 64: 981–985.

288 van Genderen PJJ, Budde U, Michiels JJ, van Strik R, van Vliet HHDM. The reduction in large von Willebrand factor multimers in plasma in essential thrombocythemia is related to platelet count. *Br J Haematol* 1996; 93: 962–965.

289 Mohri H, Ohkubo T. Acquired von Willebrand's syndrome due to an inhibitor of IgG specific for von Willebrand's factor in polycythemia rubra vera. *Acta Haematol* 1987; 78: 258–264.

290 van Genderen PJJ, Prins FJ, Lucas IS et al. Decreased half-life of plasma von Willebrand factor collagen binding activity in essential thrombocythemia: normalization after cytoreduction of the increased platelet count. *Br J Haematol* 1997; 99: 832–836.

291 Sultan Y, Caen JP. Platelet dysfunction in preleukemic states and in various types of leukemia. *Ann NY Acad Sci USA* 1972; 201: 300–306.

292 Cowan DH, Haut JJ. Platelet function in acute leukemia. *J Lab Clin Med* 1972; 79: 893–905.

293 Cowan DH, Graham RR, Jr., Baunach D. The platelet defect in leukemia, platelet ultrastructure, adenine nucleotide metabolism and the release reaction. *J Clin Invest* 1975; 56: 188–200.

294 Meschengieser S, Blanco A, Maugeri N et al. Platelet function and intraplatelet von Willebrand factor antigen and fibrinogen in myelodysplastic syndromes. *Thromb Res* 1987; 46: 601–606.

295 Levine PH, Katayama I. The platelet in leukemic reticuloendotheliosis. *Cancer* 1975; 36: 1353–1358.

296 Feiner AS, Myers AM, Moore GE. Leukemic reticuloendotheliosis: Loss of platelet defect after splenectomy. *JAMA* 1979; 1978; 240: 238–240.

297 Sweet DL, Golomb HM. Correction of platelet defect after splenectomy in hairy cell leukemia. *JAMA* 1979; 241: 1684.

298 Zuzel M, Cawley JC, Paton RC, Burns GF, McNicol GP. Platelet function in hairy-cell leukaemia. *J Clin Pathol* 1979; 32: 814–821.

299 Rosove MH, Naeim F, Harwig S, Zighelboim J. Severe platelet dysfunction in hairy cell leukemia with improvement after splenectomy. *Blood* 1980; 55: 903–906.

300 Nenci GG, Gresele P, Agnelli G, Parise P. Intrinsically defective or exhausted platelets in hairy cell leukemia? *Thromb Haemost* 1981; 46: 572.

301 Roussi JH, Houbouyan LL, Alterescu R, Franc B, Goguel AF. Acquired von Willebrand's syndrome associated with hairy cell leukemia. *Br J Haematol* 1980; 46: 503–506.

302 Lackner H. Hemostatic abnormalities associated with dysproteinemias. *Semin Hematol* 1973; 10: 125–133.

303 Vigliano EM, Horowitz HI. Bleeding syndrome in a patient with IgA myeloma: Interaction of protein and connective tissue. *Blood* 1967; 29: 823–836.

304 DiMinno G, Coraggio F, Cerbone AM et al. A myeloma paraprotein with specificity for platelet glycoprotein IIIa in a patient with a fatal bleeding disorder. *J Clin Invest* 1986; 77: 157–164.

305 Mohri H, Noguchi T, Kodama F, Itoh A, Ohkubo T. Acquired von Willebrand disease due to inhibitor of human myeloma protein specific for von Willebrand factor. *J Clin Pathol* 1987; 87: 663–668.

306 Takahashi H, Nagayama R, Tanabe Y et al. DDAVP in acquired von Willebrand syndrome associated with multiple myeloma. *Am J Hematol* 1986; 22: 421–429.

307 Mannucci PM, Lombardi R, Bader R et al. Studies of the pathophysiology of acquired von Willebrand's disease in seven patients with lymphoproliferative disorders or benign monoclonal gammopathies. *Blood* 1984; 64: 614–621.

308 Wallace MR, Simon SR, Ershler WB, Burns SL. Hemorrhagic

Cambridge Books Online © Cambridge University Press, 2009

diathesis in multiple myeloma. *Acta Haematol* 1984; 72: 340–342.
309 Hyman BT, Westrick MA. Multiple myeloma with polyneuropathy and coagulopathy. *Arch Intern Med* 1986; 146: 993–994.
310 Veyradier A, Jenkins CS, Fressinaud E, Meyer D. Acquired von Willebrand syndrome: from pathophysiology to management. *Thromb Haemost* 2000; 84: 175–182.
311 Viallard JF, Pellegrin JL, Vergnes C et al. Three cases of acquired von Willebrand disease associated with systemic lupus erythematosus. *Br J Haematol* 1999; 105: 532–527.
312 Mannucci PM, Lombardi R, Lattuada A, Perticucci E, Valsecchi R, Rumuzzi G. Supranormal von Willebrand factor multimers in scleroderma. *Blood* 1987; 73: 1586–1591.
313 Bovill EG, Ershler WB, Golden EA, Tindle BH, Edson JR. A human myeloma-produced monoclonal protein directed against the active subpopulation of von Willebrand factor. *Am J Clin Pathol* 1986; 85: 115–123.
314 Mohri H, Noguchi T, Kodama F, Itoh A, Ohkubo T. Acquired von Willebrand disease due to inhibitor of human myeloma protein specific for von Willebrand factor. *Am J Clin Pathol* 1987; 87: 663–668.
315 Mazurier C, Parquet-Gernez A, Descamps J, Bauters F, Goudemand M. Acquired von Willebrand's syndrome in the course of Waldenstrom's disease. *Thromb Haemost* 1980; 44: 115–118.
316 Stewart AK, Glynn MF. Acquired von Willebrand disease associated with free lambda light chain monoclonal gammopathy, normal bleeding time and response to prednisone. *Postgrad Med J* 1990; 66: 560–562.
317 van Genderen PJ, Vink T, Michiels JJ, van 't Veer MB, Sixma JJ, van Vliet HH. Acquired von Willebrand disease caused by an autoantibody selectively inhibiting the binding of von Willebrand factor to collagen. *Blood* 1994; 84: 3378–3384.
318 Handin RI, Martin V, Moloney WC. Antibody-induced von Willebrand's disease: a newly defined inhibitor syndrome. *Blood* 1976; 48: 393–405.
319 Goudemand J, Samor B, Caron C, Jude B, Gosset D, Mazurier C. Acquired type II von Willebrand's disease: demonstration of a complexed inhibitor of the von Willebrand factor-platelet interaction and response to treatment. *Br J Haematol* 1988; 68: 227–233.
320 Roussi JH, Houbouyan LL, Alterescu R, Franc B, Goguel AF. Acquired Von Willebrand's syndrome associated with hairy cell leukaemia. *Br J Haematol* 1980; 46: 503–506.
321 Dalton RG, Dewar MS, Savidge GF et al. Hypothyroidism as a cause of acquired von Willebrand's disease. *Lancet* 1987; 1: 1007–1009.
322 Fressinaud E, Meyer D. International survey of patients with von Willebrand disease and angiodysplasia. *Thromb Haemost* 1993; 70: 546.
323 Scott JP, Montgomery RR, Tubergen DG, Hays T. Acquired von Willebrand's disease in association with Wilm's tumor: regression following treatment. *Blood* 1981; 58: 665–669.
324 Castaman G, Lattuada A, Mannucci PM, Rodeghiero F. Characterization of two cases of acquired transitory von Willebrand syndrome with ciprofloxacin: evidence for heightened proteolysis of von Willebrand factor. *Am J Hematol* 1995; 49: 83–86.
325 Kreuz W, Linde R, Funk M et al. Induction of von Willebrand disease type I by valproic acid. *Lancet* 1990; 335: 1350–1351.
326 Bovill EG, Ershler WB, Golden EA, Tindle BH, Edson JR. A human myeloma-produced monoclonal protein directed against the active subpopulation of von Willebrand factor. *Am J Clin Pathol* 1986; 85: 115–123.
327 Mohri H, Hisanaga S, Mishima A, Fujimoto S, Uezono S, Okubo T. Autoantibody inhibits binding of von Willebrand factor to glycoprotein Ib and collagen in multiple myeloma: recognition sites present on the A1 loop and A3 domains of von Willebrand factor. *Blood Coagul Fibrinol* 1998; 9: 91–97.
328 Igarashi N, Miura M, Kato E et al. Acquired von Willebrand's syndrome with lupus-like serology. *Am J Pediatr Hematol Oncol* 1989; 11: 32–35.
329 Rao KP, Kizer J, Jones TJ, Anunciado A, Pepkowitz SH, Lazarchick J. Acquired von Willebrand's syndrome associated with an extranodal pulmonary lymphoma. *Arch Pathol Lab Med* 1988; 112: 47–50.
330 Levesque H, Borg JY, Cailleux N et al. Acquired von Willebrand's syndrome associated with decrease of plasminogen activator and its inhibitor during hypothyroidism. *Eur J Med* 1993; 2: 287–288.
331 Aylesworth CA, Smallridge RC, Rick ME, Alving BM. Acquired von Willebrand's disease: a rare manifestation of postpartum thyroiditis. *Am J Hematol* 1995; 50: 217–219.
332 Tefferi A, Nichols WL. Acquired von Willebrand disease: concise review of occurrence, diagnosis, pathogenesis, and treatment. *Am J Med* 1997; 103: 536–540.
333 Joist JH, Cowan JF, Zimmerman TS. Acquired von Willebrand's disease. Evidence for a quantitative and qualitative factor VIII disorder. *N Engl J Med* 1978; 298: 988–991.
334 Macik BG, Gabriel DA, White GCd, High K, Roberts H. The use of high-dose intravenous gamma-globulin in acquired von Willebrand syndrome. *Arch Pathol Lab Med* 1988; 112: 143–146.
335 White LA, Chisholm M. Gastro-intestinal bleeding in acquired von Willebrand's disease: efficacy of high-dose immuno-globulin where substitution treatments failed. *Br J Haematol* 1993; 84: 332–334.
336 Rinder MR, Richard RE, Rinder HM. Acquired von Willebrand's disease: a concise review. *Am J Hematol* 1997; 54: 139–145.
337 van Genderen PJJ, Terpstra W, Michiels JJ, Kapteijn L, van Vliet HHDM. High-dose intravenous immunoglobulin delays clearance of von Willebrand factor in acquired von Willebrand disease. *Thromb Haemost* 1995; 73: 890–892.
338 van Genderen PJJ, Papatsonis DNM, Michiels JJ, Wielenga JJ, Stibbe J, Huikeshoven FJM. High-dose intravenous immunoglobulin therapy for acquired von Willebrand disease. *Postgrad Med J* 1994; 70: 916–920.

Cambridge Books Online © Cambridge University Press, 2009

Platelet storage and transfusion

Scott Murphy, MD

Chief Medical Officer, American Red Cross Blood Services, Penn-Jersey Region and Department of Medicine, University of Pennsylvania, Philadelphia, USA

Introduction

Platelet transfusion has become a routine component of modern medical care. Each year, approximately 2 000 000 such transfusions are administered in the United States[1]. This chapter will review the preparation, storage, clinical use and complications of platelet transfusion.

Platelet preparation

Platelet concentrates (PC) for transfusion may be obtained from routine donations of whole blood or by apheresis (AP–PC), using citrate as the anticoagulant. There are two methods for preparing PC from whole blood, the platelet-rich-plasma method (PRP–PC) and the buffy coat method (BC–PC)(Fig. 46.1).

In evaluating the quality of PC, much attention is now being given to the number of contaminating leukocytes as well as to the appropriate platelet content. Problems related to contaminating leukocytes will be discussed in the section on complications of platelet transfusion. Many now recommend a totally leuko-reduced blood supply to reduce the frequency of these complications[2]. Platelets can be filtered during infusion at the bedside, but, for reasons to be discussed, it is probably preferable to perform leuko-reduction at the time of preparation of the PC. In the United States, an AP–PC or a pool of PRP–PC is considered leukoreduced if it contains less than 5×10^6 leukocytes while the standard in Europe is 1×10^6.

Whole-blood-derived PC

PRP–PC

At present, this is the only method used in North America for preparing whole-blood-derived PC. 450–500 ml (a unit) of whole blood is held for up to 8 hours at room temperature, and PRP is separated from red cells and buffy coat by low-speed centrifugation. The PRP is then centrifuged rapidly to produce a platelet pellet which is allowed to 'rest' for 1–2 hours before resuspension in approximately 50 ml autologous citrated plasma. Without this 'rest' period, platelets tend to clump irreversibly upon resuspension. The separated red cells are used for transfusion, while the supernatant plasma is used for transfusion or fractionation.

An average unit of PRP–PC should contain 0.8×10^{11}–0.9×10^{11}, platelets but the range around the average is high, 0.4×10^{11}–1.8×10^{11} [3,4]. One unit is adequate only for the transfusion of a small child less than 30 pounds in weight. To transfuse adults, 4 to 8 units are pooled to provide a therapeutic dose. Because of the wide range around the mean, one must pool five units to be certain that the pool will contain at least 3×10^{11} platelets[4]. Because each unit contains 0.1×10^9–0.5×10^9 leukocytes, predominantly lymphocytes, such pools contain 0.4×10^9–4.0×10^9 leukocytes, three orders of magnitude higher than a leukoreduced transfusion. There is a system which inserts a leukocyte-reduction filter between the primary blood bag and the bag that accepts the PRP[5,6]. Thus, the PRP is leukoreduced at the time of its preparation. This system was introduced in early 1998 for the preparation of all PRP–PC in Canada and is gaining acceptance in the United States.

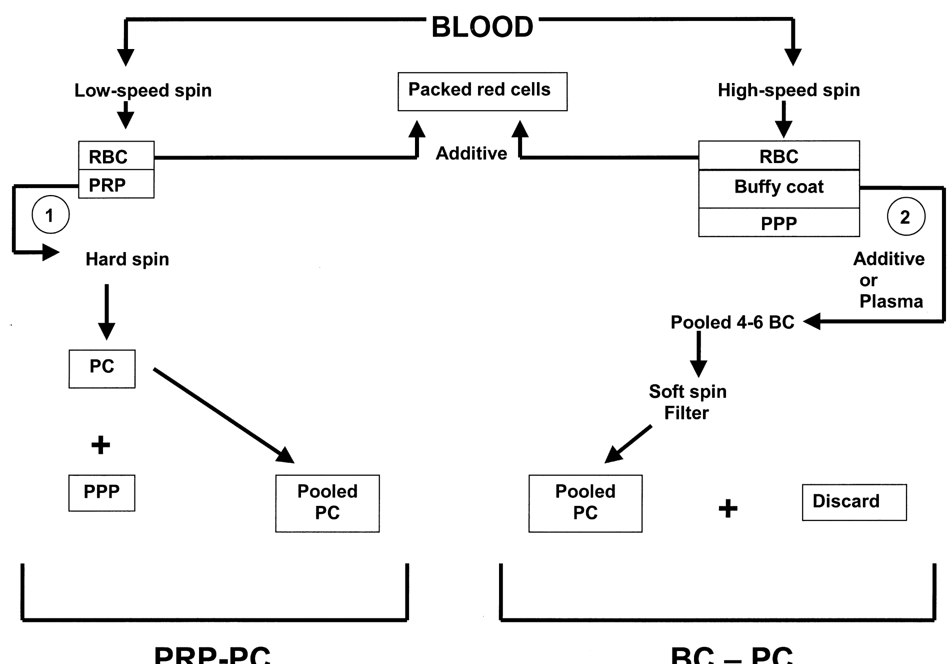


Fig. 46.1. Two ways to prepare a pool of platelet concentrates (PCs) from units of whole blood. Pathway 1 is the platelet-rich-plasma (PRP) method, the only method used in North America. Pathway 2 outlines the buffy coat (BC) method which is widely used in Europe. Both methods are described in the text. RBC, red blood cells; PPP, platelet-poor-plasma.

BC–PC

The BC method is being used with increased frequency in Europe[7]. An initial hard centrifugation sediments all blood cells so that the plasma, buffy coat, and red cells can be collected in three separate containers. The platelets at the top of the bag fall to the BC, and, remarkably, the platelets at the bottom of the bag rise to the buffy coat. Therefore, platelet yields in the buffy coat are excellent. One can pool 4–6 BCs, add twice the volume of plasma or an additive solution, centrifuge the pool at low speed to remove red cells and leukocytes, and push the supernatant through a leukoreduction filter to produce a therapeutic, leukoreduced, dose of platelets for an adult[8].

The PRP and BC methods each have their advantages and disadvantages[7,9]. Each produces platelets of high quality, and platelet yields from a unit of blood are equivalent. In the BC method, 20–25 ml of red cells are lost with the BC but an extra 70–80 ml of plasma can be collected.

Apheresis PC

One can obtain a therapeutic dose of platelets for 1–3 adults by apheresis of donors over 1–2 hours using a variety of devices[10–13] with an extraordinarily high level of safety for the donor[14]. The number of platelets obtained during the procedure varies with the platelet concentration in the blood of the donor, the volume of blood processed and the efficiency of the device. The efficiency of the newest devices is such that one should expect to obtain at least 60% of the platelets which pass through them, and most procedures are limited to 90–120 minutes. Therefore, it is the wide range of the platelet concentration in the blood of normal donors, 150000–500000 per mm^3, which accounts for the wide range in platelet yields which may be observed in practice[15]. There are also devices for obtaining an AP–PC, a unit of red cells, and a unit of plasma from the same donor at the same sitting[16].

Current standards of the Food and Drug Administration (FDA) in the United States state only that 75% of AP–PC must contain more than 3.0×10^{11} platelets. This standard probably reflected the capabilities of the apheresis devices available at the time the standard was established rather than the needs of the wide variety of patients being treated. Now, in many centres, more than 95% of collections contain 3.0×10^{11} platelets and some contain more than 10.0×10^{11} platelets. In a subsequent section, we will discuss current controversies concerning the appropriate dose for platelet transfusion. While 2.5×10^{11}–3.5×10^{11} is

Cambridge Books Online © Cambridge University Press, 2009

probably a satisfactory dose for the prophylactic transfusion of a child or small adult, it may well be insufficient for a large adult who is bleeding or has other clinical features which interfere with an optimal response to platelet transfusion. On the other hand, administration of very high yield products to small adults and children may be wasteful. Therefore, blood centres are in the process of thinking through the best way to handle the manufacturing process for apheresis platelets. Many divide high yield products (greater than 6×10^{11}–7×10^{11} platelets) to provide a therapeutic dose for two patients. Very high yield products ($>10\times10^{11}$–11×10^{11} platelets) can be divided to treat three patients. In addition, consideration is being given to the preparation of products containing two or more levels of platelet content, perhaps means of 3.2×10^{11} and 6.4×10^{11} (i.e. approximately 4 and 8 whole-blood-derived units respectively). The use of the products could be tailored to the needs of individual patients.

Platelets in PRP–PC, BC–PC, and AP–PC are probably equivalent therapeutically. However, there are two often-quoted advantages for AP–PC. First, the number of donors to whom the patient is exposed is substantially reduced thus reducing the likelihood of transmission of viral and other infectious bacterial diseases. Considering the current level of safety of the blood supply, it has been argued that choosing AP–PC for this purpose is not cost-effective[17]. Secondly, the separation technology of the various apheresis devices lends itself to the production of leukoreduced products during collection. Progressive improvements of those most recently available[10,11] has allowed the production of products with less than 1×10^6 leukocytes close to 100% of the time. However, as noted above, there are methods of leukoreduction for PRP–PC and BC–PC at the time they are manufactured. Thus, there is ongoing controversy concerning the choice between AP–PC and whole-blood-derived PC[18].

Storage of platelet concentrates

In assessing methods of platelet storage, the greatest stress has been placed on showing that the stored platelets can circulate in vivo after transfusion. The ultimate test has been the measurement of increments in platelet concentration in the blood of thrombocytopenic recipients, but reliance has also been placed on autologous, radiolabelled reinfusion studies in normal volunteers[19]. Some in vitro tests to be discussed have shown good correlation with in vivo results[20].

Liquid storage at 20–24 °C

Both whole-blood-derived and AP–PC may be stored for 5 days using the same principles: (i) The temperature must be 20–24 °C[21]; (ii) The storage container must be constructed of a plastic material that allows adequate diffusion of oxygen through its walls to meet the cells' metabolic needs[22,23]; (iii) The PC must be agitated during storage[22,23].

Using radiolabelling of stored platelets, survival after reinfusion in vivo is nearly normal if storage is carried out at 20–24 °C. However, at colder temperatures, the cells undergo irreversible disc-to-sphere transformation and survival is dramatically shortened[21]. If oxygen influx is inadequate at 20–24 °C, the cells will increase their production of lactic acid in an effort to maintain ATP levels. This leads to depletion of bicarbonate, the major buffer in plasma and fall in pH to less than 6.2[22,23]. These acid conditions lead to irreversible disc-to-sphere transformation and render the cells non-viable. A similar increase in lactic acid production and fall in pH occurs if the PC are not agitated[23]. However, agitation may be discontinued for up to 24 hours of the 5-day storage interval without harm to the platelets[24].

As already mentioned, synthetic media are now used for the storage of BC–PC[8,25,26] and they may well be used soon for apheresis PC as well[27]. Definition of the optimal solution is still in progress, but it appears that it can be relatively simple relying on 20–40% residual plasma and the presence of acetate. During storage in plasma, platelets oxidize free fatty acids in the tricarboxylic acid (TCA) cycle generating CO_2 which can leave the platelet suspension through the walls of the plastic container (Fig. 46.2). In a storage medium, acetate can enter the TCA cycle replacing the requirement for fatty acid. The oxidation of acetate utilizes a proton from the medium, thus providing an alkalinizing effect eliminating the need for bicarbonate in the medium.

Some authors have been unable to find any practical difference between fresh and stored platelets[28,29], but most find a reduction in in vivo recovery and survival of approximately 20–25% after 5 days of storage as judged by radiolabelling studies and by the increase in platelet concentration in the blood of thrombocytopenic patients[30]. Furthermore, some authors have reported an even greater defect in stored platelets relative to fresh platelets in sick patients with fever, sepsis, splenomegaly, and disseminated intravascular coagulation[31,32].

Platelet viability is no less satisfactory after 7 days of storage than after 5 days[33]. However, when storage was extended to 7 days, bacterial overgrowth and clinical sepsis in recipients of stored platelets occurred with sufficient

Cambridge Books Online © Cambridge University Press, 2009

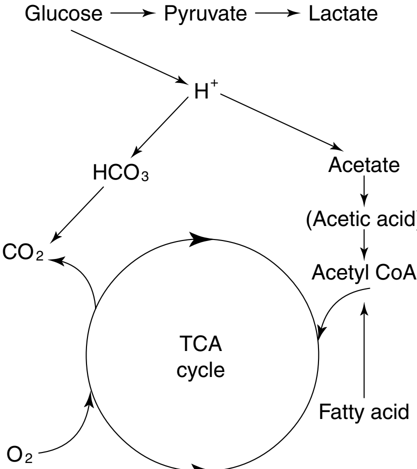


Fig. 46.2. Metabolic pathways and the control of pH during platelet storage at 20–24 °C. Almost all glucose used passes through pyruvate to form lactate and protons (H^+). Little, if any, pyruvate is decarboxylated to acetyl CoA. Rather, fatty acid provides the acetyl CoA for oxidation in the tricarboxylic acid (TCA) cycle. During storage in plasma, protons are buffered by bicarbonate. CO_2 from this source and the TCA cycle leaves the PC through the walls of the container. If there is inadequate oxygen or agitation, glycolysis accelerates overwhelming bicarbonate stores leading to fall in pH. When incorporated into a synthetic medium, acetate can bind a proton and enter the TCA cycle. This provides an alkalizing effect eliminating the need for bicarbonate in the medium.

frequency to warrant limiting liquid storage to five days[34]. If methods of bacterial decontamination[35] or bacterial detection are developed[36], it may be possible to prolong storage beyond 5 days once again.

Assuming, as most do, that platelets deteriorate to some extent during storage even under optimal circumstances, what is the nature of the platelet storage lesion? Some have proposed that it results from platelet activation[37]; others suggest that it is a form of apoptosis[38]. Either would lead to the exposure of phosphatidyl serine on the platelet surface[39]. Whatever the cause or causes of the platelet storage lesion, easily measured in vitro characteristics which correlate best with capacity to circulate in vivo are retention of disc shape and good function in the hypotonic shock response[40]. Platelets with normal discoid morphology generally circulate normally after transfusion. Platelets which are damaged by cold, acid conditions, or bacterial contamination generally lose their discoid morphology and become spheres. Normal discoid morphology is reflected by the 'swirling' or 'shimmering' appearance of well-preserved PC during gross, visual inspection[41,42]. Blood bank and clinical personnel are urged to check PC for this phenomenon prior to transfusion.

Frozen storage

The most widely studied method for frozen storage employs controlled rate freezing (1 °C per minute), 5% dimethyl sulfoxide (DMSO) as a cryoprotective agent, rapid thawing, graded reduction of the DMSO concentration, and washing prior to infusion. In vivo viability is approximately 40–50% relative to fresh platelets[43] but enhanced in vivo function has been claimed[44]. Most find that this technology is both more complex and expensive and less effective than liquid storage at 20–24 °C[45]. However, these preparations can be effective clinically and may be used for autologous transfusion of selected patients. Platelets may be obtained before myelosuppressive therapy, frozen, and then administered during subsequent periods of thrombocytopenia[46]. Newer approaches using second-messenger effectors may allow the use of lower concentrations of DMSO which would, in turn, allow the direct infusion of platelets after thawing[47].

Experimental approaches and platelet substitutes

Current methods of platelet storage are cumbersome; the duration of permissible storage is short; and 20–24 °C favours bacterial overgrowth. It would be ideal to find ways to protect platelets against the damaging effects of cold temperatures or to have a dried preparation with long shelf-life that one could simply rehydrate and infuse. A great deal of research is going on in this area examining platelets treated with paraformaldehyde and lyophilization, lyophilized platelet membrane microvesicles, fibrinogen-coated beads, albumin microspheres, and others[48]. This is an important area, but all of these ideas await validation in appropriate clinical trials.

There are also non-transfusional drugs that may help to stop thrombocytopenic bleeding. Antifibrinolytic amino acids such as aminocaproic acid and tranexamic acid would not be thought of as 'platelet substitutes'. However, they have been described as effective in controlling mucosal and dental bleeding in thrombocytopenic patients without increasing the platelet concentration in the blood[49].

Cambridge Books Online © Cambridge University Press, 2009

Clinical response to platelet transfusion

Patients with thrombocytopenia secondary to bone marrow failure

The majority of platelet transfusions are given to patients with thrombocytopenia due to decreased platelet production secondary to bone marrow disease or myelosuppressive treatment. Normally circulating platelets are in equilibrium with a pool of platelets in the spleen with one in the spleen for every two in the circulation[50]. Therefore, one-third of infused platelets will be pooled reversibly in a spleen of normal size. The fraction may be much larger if the spleen is enlarged. Assuming that the recipient's blood volume is 2.5 litres/m^2 body surface area (BSA), the infusion of one unit of whole-blood-derived PC, containing 0.8 $\times 10^{11}$ platelets, should result in an increase in platelet concentration in the blood of 21 000 per mm^3 in a recipient with 1m^2 BSA. The response to one unit will be inversely proportional to the patient's size expressed as the BSA. Thus, one can evaluate the response to a platelet transfusion by calculating the corrected count increment or CCI[51]:

$$\frac{\text{Measured increase in platelet concentration} \times \text{BSA(m}^2\text{)}}{\text{Number of units infused (or number of platelets} \times 10^{11}\text{)}}$$

The measurement of CCI has been criticized[52], but it is the most widely used method for assessing response. In practice, it can be measured 10 minutes to a few hours after transfusion[53]. Under optimal circumstances, the response should be 21 000 per mm^3 per m^2 per unit infused or 26 000 per mm^3 per m^2 per 10^{11} platelets infused.

In practice, in patients with thrombocytopenia secondary to marrow failure, the average CCI is approximately one half of that prediction, 10 000 per mm^3 per m^2 per unit infused[51]. Many studies have attempted to identify the factors responsible for this less than optimal response in real life[32,51,54–58]. Alloimmunization has been incriminated along with a variety of non-immune factors such as platelet storage, bacterial sepsis, concomitant use of antibacterial antibiotics and Amphotericin B, graft-vs-host disease, splenomegaly, disseminated intravascular coagulation, and simply having had a recent allogeneic bone marrow transplantation. It is of interest that no one factor predominates in the majority of studies, suggesting that the crucial factors vary with the populations of patients being studied. Often, none of these factors are present but the response is mediocre. It is likely that other factors, as yet undefined, are at work as well.

After a transfusion, the time until the platelet concentration returns to baseline (time to next transfusion) also varies with the immune and nonimmune factors affecting

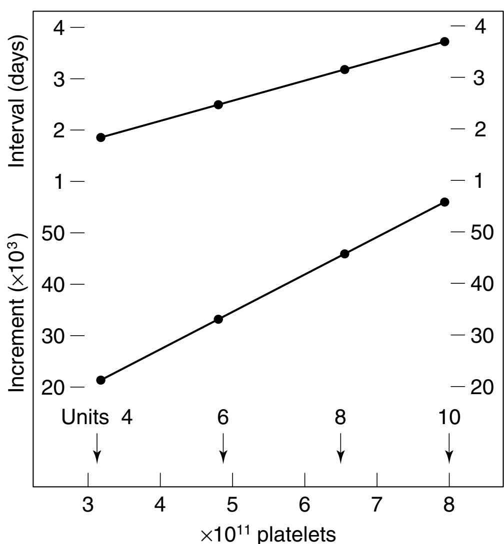


Fig. 46.3. Average response to platelet transfusion, 2 m^2 adult patient. The expected immediate increment in platelet concentration in the blood and the time for the platelet concentration to return to baseline (transfusion interval) are given relative to the platelet dose. Units, whole-blood-derived PC.

the initial CCI[59]. Furthermore, platelet survival is reduced in all patients with thrombocytopenia, regardless of the cause, with progressive reduction as the platelet concentration in the blood becomes lower[60]. Therefore, time to next transfusion varies directly with the height of the platelet concentration achieved by the transfusion and, therefore, the dose of platelets that was administered[61–63]. Everything else being equal, the larger the dose, the higher the platelet concentration achieved by transfusion, and the longer the time will be to the next transfusion (Fig. 46.3).

Platelet dose

If, as described above, the average patient has an increase in platelet concentration of 10 000 per mm^3 per m^2 per unit of platelets transfused, one can calculate the relationship between the dose administered and the average rise in the platelet concentration to be expected. Furthermore, using calculations and data[60–63], one can estimate the average time to the next transfusion. For example, for the transfusion of a myelosuppressed patient with a 2 m^2 surface area these values are given in Fig. 46.3. If the patient was not bleeding, was being transfused prophylactically, and was hospitalized so that he could be transfused again 48 hours later, five units of whole-blood-derived platelets or 4×10^{11} apheresis platelets would probably be satisfactory since raising the platelet concentration by 20 000–30 000 per

Cambridge Books Online © Cambridge University Press, 2009

mm^3 protects most thrombocytopenic patients against spontaneous, catastrophic bleeding, and there would be a 2-day transfusion interval. On the other hand, for the doses given in Fig. 46.3, the platelet concentrations achieved in a 1 m^2 patient would be twice those in Fig. 46.3 and lower doses would probably be satisfactory.

However, ten units (8.0×10^{11}) or more would be a better choice for the 2 m^2 patient if the goal was to achieve a platelet concentration over 50 000 per mm^3 because the patient was bleeding or was being prepared for an invasive procedure.[64] It is probably most critical to achieve this higher level of platelet concentration if the surgical field is highly vascular, as with inflammation or portal hypertension; if there are coexisting defects in plasma coagulation; if the procedure is 'blind' as in needle biopsy of the liver, when there is no opportunity to achieve hemostasis mechanically; or if the surgery is in an area where even a small hemorrhage could be disastrous, as in the central nervous system. A higher dose might also be chosen to facilitate transfusion in the outpatient setting where a longer transfusion interval would be preferable. However, patients vary dramatically in their responses. Therefore, the measurement of increments 1 and 24 hours after transfusion is a cost-effective way to modify the dose and frequency of transfusion based on the pathophysiology of the individual patient.

All of these considerations stress that no single dose meets the needs of all patients. On the other hand, one could argue for using the highest possible dose in all patients since increments are higher and fewer episodes of transfusion are required because of the longer interval between transfusions. However, theoretical considerations[61] and patient data[65] suggest that more platelets are utilized with such a strategy than with frequent, low-dose transfusions.

Platelet transfusion trigger

A thrombocytopenic patient who is actively bleeding requires platelet transfusion. It is more difficult to decide about transfusion if the platelet count is simply very low and the patient has no hemorrhagic signs or only minor ones such as petechiae or small ecchymoses of the skin. Clinical experience suggests that, if the platelet count is low enough, long enough, 'spontaneous', major hemorrhage, particularly into the central nervous system, may occur. Unfortunately, in an individual patient, we do not know how low and how long.

Studies of patients with acute leukemia carried out 40 years ago, prior to the availability of platelet transfusion, described the relationship between platelet concentration in the blood and clinical hemorrhage[66]. Minor and major hemorrhage began when the platelet concentration fell below 50 000 and 20 000 per mm^3, respectively. Major hemorrhage was observed in the range, 5 000–20 000 per mm^3, but on only 3% of patient days. There was no cut-off between bleeding and non-bleeding patients in that range, but there was a rapid increase in the rate of major bleeding when the platelet concentration fell below 5 000 per mm^3 reaching a frequency of 33% of patient–days as the platelet concentration approached 0 per mm^3.

Subsequently, the same group described the effect of prophylactic platelet transfusion administered whenever the platelet concentration fell below 20 000 per mm^3 [67]. There was a striking reduction in major hemorrhage when the platelet concentration (measured pretransfusion) was below 5 000 per mm^3 but no substantial change in the range, 5 000–20 000 per mm^3. Nonetheless, for many years, this experience was used to justify prophylactic platelet transfusion whenever the platelet concentration fell below 20 000 per mm^3, although the data could have been used to support 5,000 per mm^3 as an appropriate trigger.

More recently, prospective but uncontrolled studies by one group supported the safety and efficacy of a more restrictive policy using 5 000 per mm^3 as the platelet transfusion trigger[68,69]. Subsequently, three prospective studies assigned patients to one of two groups receiving prophylactic platelet transfusion at either 10 000 per mm^3 or 20 000 per mm^3 [70–72]. Uniformly, there was no increase in bleeding risk at the lower transfusion trigger which is now being adopted by many transfusion services[73].

However, it is likely that it is just as unjustified to choose a rigid, unvarying transfusion trigger as it is to choose a rigid, unvarying transfusion dose. Clinical factors may increase the risk of hemorrhage at any given platelet concentration. These include fever and sepsis, administration of drugs that interfere with platelet function, coexistent abnormalities of plasma coagulation factors, and high leukocyte concentrations in the blood. It is appropriate to raise the transfusion trigger in complicated, clinically ill patients of this type.

In addition, moderate to severe bleeding is observed in 11–23% of patients after bone marrow transplantation in spite of the aggressive use of prophylactic platelet transfusion and maintenance of morning platelet counts above 20 000 per mm^3 [74,75]. The types of bleeding are most commonly gastrointestinal and urinary and less commonly pulmonary and intracranial. Usually, there is an identifiable anatomic cause such as gastrointestinal ulceration, hemorrhagic cystitis, or diffuse alveolar hemorrhage. In effect, it is common in the myelosuppressed patient to be treating bleeding, not preventing it. Many

Cambridge Books Online © Cambridge University Press, 2009

centres increase the transfusion trigger to 30 000 or even 50 000 per mm^3 in such cases just as one would prior to a surgical procedure[74].

Thrombocytopenia due to platelet loss, sequestration, or destruction

As described in the previous section, most patients receiving platelet transfusion have a defect in platelet production. However, many patients with other types of thrombocytopenia and platelet dysfunction are transfused as well.

Massive transfusion of red cells

Dilutional thrombocytopenia will occur when massive blood loss is replaced with units of stored red cells which lack viable platelets. Following replacement of one blood volume, 35–40% of the original platelets usually remain. Even when one to two blood volumes have been replaced, abnormal bleeding usually does not develop, and routine transfusion is not indicated simply because the platelet concentration in the blood is low[76]. Platelets should be given to patients who demonstrate abnormal bleeding.

Cardiopulmonary bypass

Immediately following and for several days after cardiopulmonary bypass, the platelet concentration commonly falls to subnormal levels, occasionally as low as 50 000 per mm^3. There is an associated defect in platelet function. Prospective studies have shown no benefit from the prophylactic administration of platelet transfusions to such patients[77]. Just as in the case of massive transfusions, they should be reserved for the relatively rare patient who demonstrates clinically abnormal bleeding.

Splenomegaly

As mentioned previously, patients with massive splenomegaly have thrombocytopenia related predominantly to excessive sequestration in a splenic pool in continuous exchange with platelets in the circulation[50]. The platelet concentration rarely falls below 30 000 per mm^3 due to this mechanism alone, so that platelet transfusion is rarely considered except in anticipation of invasive procedures. Depending on the degree of splenic enlargement, one may have to administer 10 to 15 units of whole-blood-derived PC per m^2 body surface area to achieve a substantial increase in platelet concentration. In many patients with very marked splenomegaly, it may not be possible to achieve platelet concentration elevations even with very large doses of PC.

Idiopathic thrombocytopenic purpura (ITP)

In ITP, platelet transfusion is generally not used because the bleeding tendency is less severe than in thrombocytopenia due to diminished production, and the response to medical therapy is generally satisfactory and rapid (see Chapter 4). Furthermore, the survival of transfused platelets is relatively brief, similar to that of the patient's own platelets. Nonetheless, when there is critical bleeding or need for urgent surgery, platelet transfusion will generally raise the platelet count substantially for 12 to as long as 48 hours[78]. The same general principles apply to other diseases in which there is accelerated destruction of platelets, such as disseminated intravascular coagulation.

Neonatal alloimmune thrombocytopenia

In this syndrome, the mother produces an alloantibody against antigens on fetal platelets that have crossed the placenta (Chapter 6). The antibody, in turn, crosses the placenta, causing in utero thrombocytopenia, beginning as early as 20 weeks gestation. Intracerebral hemorrhage may occur in utero. The thrombocytopenia may persist for weeks after delivery. The most common antibodies is to HPA-1a (Pl^{A1}) but antibody to other platelet antigens may be involved as well.

It is common for this disease to present with the delivery of a severely thrombocytopenic newborn at the end of a first pregnancy to the surprise of the attending physicians. It is optimal to transfuse the newborn with platelets which lack the antigen to which the antibody has been formed. Since maternal platelets are compatible, platelets harvested from the mother's blood by apheresis can produce an increase in the platelet concentration of the blood of the infant (Fig. 46.4)[79]. Ideally, such platelets should be concentrated in a small volume of plasma to avoid infusing additional antibody.

Unfortunately, it is often difficult to arrange for apheresis of the mother. A randomly selected unit of platelets may raise the neonate's platelet concentration substantially[80]. If it does not, prompt serologic evaluation of the mother and father can identify the antigen to which the antibody has been formed. If the antibody is directed against HPA-1a (Pl^{A1}), platelets lacking HPA-1a (i.e. homozygous HPA-1b) can be used to support the infant[81]. One must be certain that the mother has not also formed antibody to HLA antigens which might be present on the donor's platelets[82].

Cambridge Books Online © Cambridge University Press, 2009

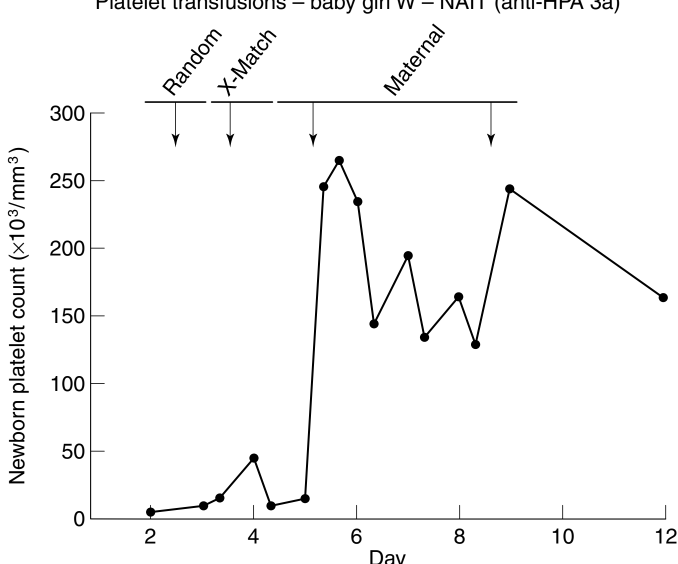


Fig. 46.4. Platelet transfusions in newborn with neonatal alloimmune thrombocytopenia secondary to anti HPA3a, BAK[a]. There was poor response to randomly selected and cross-matched platelets from unrelated donors. Platelets from the mother provided an excellent response on two occasions.

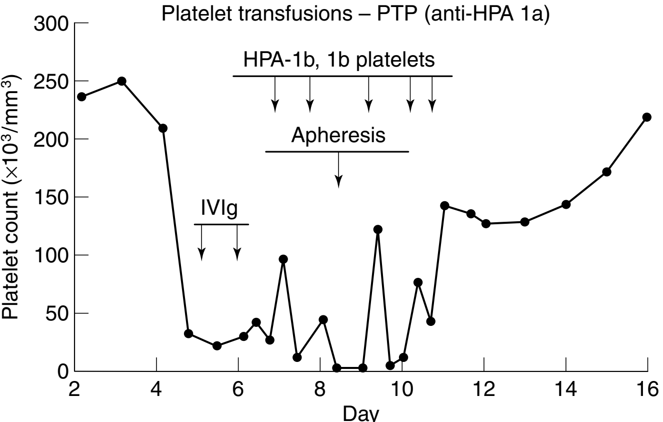


Fig. 46.5. Platelet transfusions in women with post-transfusion purpura associated with anti HPA1a, Pl[A1]. The patient developed severe thrombocytopenia and subarachnoid hemorrhage 8 days after blood transfusion during an aortic aneurysm repair. Randomly selected platelets produced severe rigors, chills, and hypertension. On five occasions, she had no adverse reactions and good increments in platelet concentration after transfusion with platelets from Pl[A1] negative (HPA-1b homozygote) donors. Data courtesty of Dr Alison Wakoff.

Should there be a subsequent pregnancy, the recurrence rate is high (>85%). Thus, there may be further requirements for platelet transfusion of the fetus in utero to prevent serious bleeding before delivery[83,84]. Some protocols call for weekly fetal blood sampling beginning at 20 weeks gestation with transfusions of platelets to the fetus each time. Variations of the normal apheresis procedures can be used to produce highly concentrated platelets from either the mother or a normal donor who lacks the antigen to which the antibody has formed[84].

Post-transfusion purpura

In this complex illness (Chapter 7), shortly after red cell transfusion, the patient develops an alloantibody to a platelet antigen (generally HPA-1a, Pl[A1]) on the platelets present in the transfused red cells. Autologous platelets lacking that platelet antigen are destroyed as well by mechanisms which are poorly understood. Random platelet transfusions are thought to be ineffective and may cause severe febrile and anaphylactoid reactions. HPA-1a-negative platelets may give some benefit[85]. Figure 46.5 provides an example of a patient where that seemed to be the case.

Hereditary thrombocytopenia

These syndromes are rare and generally not associated with severe bleeding (Chapter 2). Since the survival of allogeneic platelets is normal, platelet transfusion is quite effective and may be used for critical bleeding and surgery.

Qualitative platelet disorders

In spite of normal platelet counts, patients with qualitative platelet disorders have a clinical bleeding tendency associated with abnormal in vitro tests of platelet function and a prolonged bleeding time in vivo. The basis may be hereditary or acquired (Chapters 10–13). Platelet transfusion is generally not indicated when the cause is extrinsic to the platelet, as in uremia, von Willebrand disease, and hyperglobulinemia, since the transfused platelets will function no better than the patient's own platelets. There are exceptions in certain types of von Willebrand's disease in which normal platelets can be used to deliver their content of von Willebrand factor to a bleeding site[86]. Most inherited intrinsic disorders are mild and do not require platelet transfusions even for surgery if the procedure is carried out under direct vision, so that hemostasis may be achieved mechanically. If the bleeding tendency is more severe, as in

Cambridge Books Online © Cambridge University Press, 2009

thrombasthenia, platelet transfusions may be necessary for more severe bleeding and surgery. The acquired defects, as in the myeloproliferative and myelodysplastic syndromes, generally do not require platelet transfusion unless there is coexistent thrombocytopenia.

Possible contraindications to platelet transfusion

Concern has been voiced that platelet transfusions should not be administered to patients with forms of thrombocytopenia associated with thrombosis such as thrombotic thrombocytopenic purpura (TTP) and heparin-induced thrombocytopenia (Chapters 8, 9). It is reasoned that platelets participate in thrombus formation and that transfusions may precipitate deterioration of the disease; there are anecdotal reports of such events[87,88]. However, particularly in TTP, platelet transfusion is often requested prior to invasive procedures such as the insertion of intravenous catheters for therapy with apheresis or in response to severe clinical bleeding. The author's experience has been that platelet transfusions are safe in this setting. However, it seems prudent not to administer prophylactic platelet transfusions simply because the platelet concentration is low in these diseases.

Complications of platelet transfusion – prevention and management

There are many complications of platelet transfusion (Table 46.1). Paradoxically, the majority are not due to the platelets themselves but rather to contaminating leukocytes, red cells, plasma proteins, and microorganisms.

Complications due to contaminating leukocytes

Alloimmunization to Class I HLA antigens

Human leukocyte antigens (HLA) are expressed on integral membrane glycoproteins. Almost all cells have Class I antigens (A, B, and C subloci), whereas only a few types of circulating leukocytes (dendritic cells, monocytes, and subsets of B cells) have Class II antigens. Primary alloimmunization to Class I HLA antigens appears to require presentation of such antigens on cells that also express Class II antigens and other costimulatory molecules[89]. There is now abundant evidence that the incidence of HLA alloimmunization can be reduced by the consistent use of leukoreduced blood products[90,91].

Table 46.1. Complications of platelet transfusion

Due to contaminating leukocytes
Alloimmunization to Class I HLA antigens
Refractoriness to platelet transfusion
Febrile nonhemolytic transfusion reactions (FNHTR)
Cytokine formation
FNHTR
Transmission of cytomegalovirus (CMV)
Graft-vs.-host disease (GVHD)
Reactions associated with bedside leukoreduction filters
Due to contaminating red cells
Rh alloimmunization
Parasites – malaria, babesiosis
Due to plasma and its contents
Contaminating microorganisms
Bacteria
Viruses – HCV, HIV, HBV
Parasites – Chagas Disease
Plasma proteins
Allergic reactions
IgA in patients with IgA deficiency
? FNHTR
ABO antibodies
Transfusion-related acute lung injury (TRALI)
Due to platelets themselves
Alloantibodies to platelet-specific antibodies
? Allergic reactions to platelet products

However, it remains to be seen how completely leukoreduction can eliminate alloimmunization. In one study[92], leukoreduction had almost no effect on the development of alloimmunization in patients who had been previously exposed to foreign leukocytes either through pregnancy or transfusions that had not been leukoreduced. It may be that leukoreduction will prove to be less effective in preventing secondary as opposed to primary alloimmunization. However, another trial[90] showed efficacy of leukoreduction in such patients but failure in some patients who had not been previously exposed to foreign leukocytes.

Traditionally, platelet transfusions have been begun with PC from random donors and continued until HLA alloimmunization is suspected because there have been two or three consecutive platelet transfusions with CCI less than 3000 per mm^3 per m^2 per unit[93]. In the future, it may be wise to screen patients intermittently for HLA antibody so that HLA alloimmunization can be detected prior to refractoriness rather than vice versa. HLA alloimmunization can be confirmed in the laboratory by performing a lymphocy-

Cambridge Books Online © Cambridge University Press, 2009

totoxicity (LCT) assay for HLA antibody in the patient's serum. In this assay, leukocytes from 50–100 donors with an appropriate heterogeneity of HLA types are incubated with the patient's serum and complement. LCT is assessed microscopically. The presence of such antibody has been a good predictor of poor response to platelets from randomly selected donors[94] and improved response when platelets are HLA selected[95]. Many centres consider it inappropriate to issue platelets matched according to HLA type unless this or a similar test has demonstrated HLA antibody anticipating that matching will provide little benefit if antibody is not present[96,97]. The pattern and intensity of immunization varies greatly from patient to patient. An LCT assay can be characterized by the percentage of cells in the panel against which the patient's serum reacts, i.e. the percentage reactive antibody (PRA). Furthermore, the pattern of reactivity can be analysed to determine the antigens to which the patient has formed antibody. Patients may have PRAs at any level between 1 and 100%.

The incidence and severity of HLA alloimmunization should be gradually decreasing as the use of leukoreduced blood products becomes increasingly popular. The following discussion is based on data accumulated before widespread leukoreduction was in use. Approximately 10% of patients presenting for therapy of diseases requiring platelet transfusion will already have LCT antibodies from prior transfusions and pregnancies[98]. Another 30% become alloimmunized during therapy and 60% never do[98]. There is no known difference between those who do, and do not, become immunized. Among those who do, some will do so after only 2–4 transfusions whereas others require dozens of transfusions[99]. The majority of patients who become alloimmunized establish a level and specificity of immunization and tend to maintain that status as they continue to be transfused[100–102]. However, approximately 30% lose their antibodies over time in spite of continuing transfusion[100]. Thus, it is helpful to monitor antibody levels and specificity since such patients may regain responsiveness having been previously refractory.

The antigenic specificities of the HLA antibodies formed by these patients vary dramatically. Crossreactive groups (CREGs) of HLA antigens have been defined by serological testing. Crossreactivity among antigens in a CREG is based on the sharing of one or more public epitopes by those antigens[103]. It is common for some patients to develop antibodies to one or more public epitopes, i.e. one or more CREGs, while others develop antibodies to one or more private antigens within a CREG[104]. Furthermore, some patients demonstrate intraCREG antibodies, i.e. antibodies to antigens in the same CREGs as the patient's own antigens[105]. These facts have major implications for management.

Table 46.2. Classification of donor/recipient pairs on the basis of HLA Class I matching

A	All four antigens in donor identical to those of recipient.
B1U	Only three antigens detected in donor; all present in recipient.
B1X	Three donor antigens identical to recipient; fourth antigen cross-reactive[a] with recipient.
B2U	Only two antigens detected in donor; both present in recipient.
B2UX	Only three antigens detected in donor; 2 identical with recipient, third cross-reactive.
B2X	Two donor antigens identical to recipient; third and fourth antigens cross-reactive with recipient.
C	One antigen of donor not present in recipient and not cross-reactive with recipient.
D	Two antigens of donor not present in recipient and not cross-reactive with recipient.

Note:
[a] Antigen in a cross-reactive group (CREG) which contains one of the patient's antigens.

Management of the refractory patient

Techniques have evolved so that there are now three approaches which are useful in the management of alloimmunized patients. In 1969, it was shown that refractory patients would respond to platelets from siblings who were identical for all four Class I HLA antigens[106]. Similarly, patients could be supported by platelets from unrelated donors who were HLA-identical or closely matched. Because some patients do not make antibody to antigens within their own CREGs, it became popular to choose donors according to CREG classification, particularly BX matches, i.e. donors whose antigens are identical to or within the same CREGs as those of the patient[107]. Table 46.2 shows the categorization of such matches.

Responses were better with such matching than with random donor selection, but many BX matches failed and many C and D matches succeeded. Some patients with relatively low PRAs have antibody to only one or two CREGs so that success with some C and D matches would be expected by chance. On the other hand, failure of some BX matches would be expected in patients with intraCREG antibody. One study[108] described BX matching pairs that frequently failed and another[109] found BX matches to be no better than randomly selected platelets.

In the late 1980s and early 1990s, practical methods for platelet crossmatching became available[110]. Many centres found that they could simply crossmatch patient's serum with unselected AP-PC in inventory to find, within hours, a

Cambridge Books Online © Cambridge University Press, 2009

compatible product that would be successful in vivo[54,110]. However, it was recognized[110] that, in a highly immunized patient, one might crossmatch with dozens of donors and not find a compatible product. For many such patients, only the identification of an A or BU match (Table 46.2) will suffice.

Yet another approach has been recently proposed[101]. If the PRA in the LCT assay is less than 100%, one should be able to identify the HLA antigens to which the patient has not formed antibody. The patient can then be supported with ‘antigen-negative’ platelets, i.e. platelets which lack the antigens to which the patient has formed antibody. If the results of the LCT assay are known, one can often provide a product from inventory on an urgent basis using this approach.

There is no reason that one of these three approaches should be chosen to the exclusion of the other two[102]. If the PRA is less than 70%, successful support can usually be provided by crossmatching of random products or by ‘antigen-negative’ platelets. When the PRA is high (>80%), one can select for cross-matching the best available ‘antigen-negative’ HLA matches and/or selectively recruit A and BU matches from an HLA-typed donor file. Some patients with common HLA types, 1,2/7,8 for example, will have dozens of A and BU matches available. Unfortunately, some patients have rarer HLA types and will have no A or BU matches to recruit.

Role of ABO and platelet-specific antibodies in refractoriness to platelet transfusions

ABO determinants are carried by both glycoproteins and glycolipids of platelets, as they are on red cells[111]. Group O patients commonly have a good response to platelets from group A or B donors, but, in a subset of patients, the response can be poor[112] or very poor[113]. On the other hand, transfusing group A or B patients with platelets from group O donors exposes the recipient’s red cells to anti-A and anti-B in the donor’s plasma. Accelerated destruction of the patient’s red cells is rare[114], but frank acute hemolysis has been observed[114–116]. Furthermore, it has been proposed that formation of immune complexes between A and B substance and corresponding antibodies may have deleterious effects[117]. Thus it seems wise to observe ABO compatibility when possible.

The platelet surface carries many platelet-specific antigens which are quite capable of eliciting a strong alloantibody response as in neonatal alloimmune thrombocytopenia and post transfusion purpura. It is surprising that there are only a few case reports of such alloantibodies accounting for refractoriness to platelet transfusion[118–121]. It appears that fewer than 10% of multi-transfused patients develop platelet-specific antibody[90]; those who do also tend to form HLA antibodies.

Febrile and allergic transfusion reactions

Acute reactions to platelet transfusions are generally classified as febrile (fever, chills) or allergic (urticaria, rash, dyspnea, and bronchospasm). Prior to the availability of methods for leukoreduction, approximately 20% of platelet transfusions were accompanied by febrile reactions[122]. Some of these reactions were undoubtedly due to antibodies in the patient directed against either leukocyte-specific or HLA antigens on leukocytes contaminating the PC. Leukocyte depletion by filtration during infusion reduced the frequency of these reactions, but many continued to occur[122,123]. It is now clear that contaminating leukocytes produce inflammatory cytokines such as interleukin-1, interleukin-6, interleukin-8, and tumour necrosis factor-alpha during storage at 20–24 °C and that these compounds are responsible for many febrile reactions since they are not removed by bedside filtration[124,125]. Thus febrile reactions occur more frequently when the PC are transfused at their end of the storage interval[126,127]. These reactions provide a strong argument for routine, prestorage removal of leukocytes.

Nonetheless, febrile reactions occur in approximately 2% of platelet transfusions even with prestorage leukoreduction[128]. Furthermore, it appears that contaminating leukocytes are not involved in the production of allergic reactions[126,129]. The origins of these reactions are not known. They may be related to plasma proteins or products produced during storage by the platelets themselves. In patients who develop these difficult problems, one can wash the platelets free of plasma prior to infusion[130].

Finally, there may be complications of platelet transfusion related to the removal of leukocytes by filtration of PC at the bedside. Severe hypotension has been reported[131] predominantly when negatively charged leukocyte reduction filters are used in patients who are receiving angiotensin-converting enzyme inhibitors[132]. One proposed mechanism is that high molecular weight kininogen is converted to bradykinin, a potent vasodilator, by exposure to a negatively charged surface. Bradykinin is normally metabolized by ACE in a few seconds, but may circulate much longer in patients receiving ACE inhibitors. Although the mechanisms behind these hypotensive reactions are still hypothetical and more work needs to be done, this type of reaction provides another reason for prestorage rather than bedside leukoreduction. Prestorage leukoreduction at the blood centre offers the advantage that it is carried out under standardized conditions following good manufacturing practices (cGMP) with appropriate quality control

Cambridge Books Online © Cambridge University Press, 2009

procedures in place. Such standardization is not possible with filtration at the bedside.

Transmission of cytomegalovirus (CMV)

In asymptomatic carriers, this virus resides in the nuclei of subsets of leukocytes with little virus free in plasma. It has been shown that the use of leukoreduced blood components is essentially equivalent to the use of components from donors who have negative tests for CMV antibody in terms of risk of CMV transmission[133]. Since this infection is particularly dangerous for severely immunocompromised patients such as those who have had a recent allogeneic bone marrow transplantation, some clinicians continue to use CMV-negative blood products along with leukoreduction in this select population.

Graft-vs.-host disease (GVHD)

Immunosuppressed patients may develop graft-vs.-host disease (GVHD) from T-lymphocytes present in any transfusion, including platelet transfusion. Thus, it is standard practice to treat PC with gamma irradiation to inhibit proliferation of these T-lymphocytes when the recipient had been heavily immunosuppressed[134]. Clinicians are progressively applying this practice to all patients who have received cytotoxic chemotherapy. Exposure to 5 000 rad appears to have no deleterious effect on platelets. It is important to emphasize that current methods of leukoreduction do not remove enough T-cells to prevent GVHD.

Complications due to contaminating red cells

When transfusing PC to girls or young women who are Rh-negative, one needs to be concerned about the possibility of sensitization by Rh-positive red cells contaminating infused platelets. In practice, sensitization is rather uncommon in immunosuppressed patients[135]. However, where possible, one should administer platelets from Rh-negative donors. When this is not possible, one can administer Rh immunoglobulin (RhoGAM), about 20 microgram intramuscularly per unit of platelets, so that the infused red cells will be cleared prior to sensitization. A full dose of RhoGAM, 300 μg, is sufficient to suppress the immune response to 15 ml of Rh positive red cells.

There are enough red cells contaminating PC to transmit both malaria and babesiosis if the donor is parasitemic with these infections.

Complications due to plasma and its contents

Contaminating microorganisms

Storage of PC at 20–24 °C allows proliferation to dangerous levels of bacteria that occasionally contaminate units of whole-blood-derived or AP-PC[34]. Contamination may occur because of asymptomatic bacteremia in the donor or at the time of venipuncture because of inadequate decontamination of the skin or because of venipuncture through areas of the skin where bacterial colonization is deeper than can be reached by such decontamination. Bacterial contamination which might not be clinically significant after 2–3 days of storage may become so after 5–7 days. As previously mentioned, PC storage is limited to 5 days for this reason.

The magnitude of this problem is commonly underestimated[136]. Estimates suggest that there may be contamination of 5–10 per 10 000 whole-blood-derived PC and 150 clinical episodes associated with severe morbidity and death in the United States per year[137]. This equates to one episode of severe morbidity or mortality per 20 000 platelet transfusions which is 50- to 250-fold higher than the risk of mortality from transmission by transfusion of human immunodeficiency virus (HIV) or hepatitis B or C infection.

There are several potential approaches to this important problem. Because apheresis involves only one donor and one venipuncture, there may be less risk than for pooled whole-blood-derived PC, although this has not been conclusively demonstrated. Under investigation are methods for screening platelet products for bacteria before transfusion[36] and methods of viral inactivation which also inactivate bacteria[35]. Parenthetically, the methods of viral inactivation may inactivate T-lymphocytes and prevent GVHD as well[138].

The plasma diluent of PC can transmit viruses such as hepatitis B and C and HIV. Recently improved methods of donor screening and testing have markedly reduced but not totally eliminated this risk. Transmission of the parasite, *Trypanosoma cruzei*, which is responsible for Chagas disease has also occurred with platelet transfusion.

As with any plasma infusion, one may see urticaria, anaphylactic shock in a patient with IgA deficiency and circulating anti-IgA, and transfusion associated acute lung injury (TRALI) when a donor has leukocyte antibodies which can react with antigens on the leukocytes of the recipient[139].

Cambridge Books Online © Cambridge University Press, 2009

References

1 Wallace EL, Churchill WH, Surgenor DM et al. Collection and transfusion of blood and blood components in the United States, 1994. *Transfusion* 1998; 38: 625–636.
2 Williamson L. Leukocyte depletion of the blood supply – how will patients benefit? *Br J Haemat* 2000; 110: 256–272.
3 Kelley DL, Fegan RL, Ng AT et al. High-yield platelet concentrates attainable by continuous quality improvement reduce platelet transfusion cost and donor exposure. *Transfusion* 1997; 37: 482–486.
4 Hoeltge GA, Shah A, Miller JP. An optimized strategy for choosing the number of platelet concentrates to pool. *Arch Pathol Lab Med* 1999; 123: 928–930.
5 Devine DV, Bradley AJ, Maurer E et al. Effects of prestorage white cell reduction on platelet aggregate formation and the activation state of platelets and plasma enzyme systems. *Transfusion* 1999; 39: 724–734.
6 Sweeney JD, Kouttab NM, Penn CL et al. A comparison of prestorage WBC-reduced whole-blood-derived platelets and bedside-filtered whole-blood-derived platelets in autologous progenitor cell transplant. *Transfusion* 2000; 40: 794–800.
7 Murphy S, Heaton WA, Rebulla P. Platelet production in the old world and the new. *Transfusion* 1996; 36: 751–754.
8 de Wildt-Eggen J, Nauta S, Schrijver JG et al. Reactions and platelet increments after transfusion of platelet concentrates in plasma or an additive solution: a prospective, randomized study. *Transfusion* 2000; 40: 398–403.
9 Heaton WAL, Rebulla P, Pappalettera M, Dzik WH. A comparative analysis of different methods for routine blood component preparation. *Transfus Med Rev* 1997; 11: 116–129.
10 Elfath D, Tahhan H, Mintz P et al. Quality and clinical response to transfusion of prestorage white cell-reduced apheresis platelets prepared by use of an in-line white cell-reduction system. *Transfusion* 1999; 39: 960–966.
11 Yockey C, Murphy S, Eggers L et al. Evaluation of the Amicus separator in the collection of apheresis platelets. *Transfusion* 1998; 38: 848–854.
12 Holme S, Andres M, Goermar N, Giordano GF. Improved removal of white cells with minimal platelet loss by filtration of apheresis platelets during collection. *Transfusion* 1999, 39: 74–82.
13 Zeiler T, Zingsem J, Moog R et al. Periodic alternating interface positioning to lower WBC contamination of apheresis platelet concentrates: a multicenter evaluation. *Transfusion* 2000; 40: 687–692.
14 Despotis GJ, Goodnough LT, Dynis M et al. Adverse events in platelet apheresis donors: a multivariate analysis in a hospital-based program. *Vox Sang* 1999; 77: 24–32.
15 Goodnough LT, Ali S, Despotis G et al. Economic impact of donor platelet count and platelet yield in apheresis products: relevance for emerging issues in platelet transfusion therapy. *Vox Sang* 1999; 76: 43–49.
16 Elfath MD, Whitley P, Jacobson MS et al. Evaluation of an automated system for the collection of packed RBCs, platelets, and plasma. *Transfusion* 2000; 40: 1214–1222.
17 Lopez-Plaza I, Weissfeld J, Triulzi DJ. The cost-effectiveness of reducing donor exposures with single-donor versus pooled random-donor platelets. *Transfusion* 1999; 39: 925–932.
18 Chambers LA, Herman JH. Considerations in the selection of a platelet component: apheresis versus whole-blood-derived. *Transfus Med Rev* 1999; 13: 311–322.
19 Holme S, Heaton A and Roodt J. Concurrent labeling method with ^{111}In and ^{51}Cr allows accurate evaluation of platelet viability of stored platelet concentrates. *Br J Haemat* 1993; 84: 717–723.
20 Murphy S, Rebulla P, Bertolini F et al. In vitro assessment of the quality of stored platelet concentrates. *Transfus Med Rev* 1994; 8: 29–36.
21 Murphy S, Gardner FH. Platelet preservation. Effect of storage temperature on maintenance of platelet viability – deleterious effect of refrigerated storage. *N Engl J Med* 1969; 280: 1094–1098.
22 Murphy S. Platelet storage for transfusion. *Semin Hematol* 1985; 22: 165–177.
23 Murphy S, Gardner FH. Platelet storage at 22 °C: role of gas transport across plastic containers in maintenance of viability. *Blood* 1975; 46: 209–218.
24 Hunter S, Nixon J, Murphy S. The effect of interruption of agitation on platelet quality during storage for transfusion. *Transfusion* 2001; 141: 809–814.
25 Murphy S. The efficacy of synthetic media in the storage of human platelets for transfusion. *Transfus Med Rev* 1999; 13: 153–163.
26 Gulliksson H, Larsson S, Kumlein G, Shanwell A. Storage of platelets in additive solutions: effects of phosphate. *Vox Sang* 2000; 78: 176–184.
27 Corash L, Behrman B, Rheinschmidt M et al. Post-transfusion viability and tolerability of photochemically treated platelet concentrates (PC). *Blood* 1997; 90: 267a.
28 Shanwell A, Larsson S, Aschan J et al. A randomized trial comparing the use of fresh and stored platelets in the treatment of bone marrow transplant recipients. *Eur J Haematol* 1992; 49: 77–81.
29 Leach MR, AuBuchon JP. Effect of storage time on clinical efficacy of single-donor platelet units. *Transfusion* 1993; 33: 661–664.
30 Murphy S, Kahn RA, Holme S et al. Improved storage of platelets for transfusion in a new container. *Blood* 1982; 60: 194–200.
31 Peter-Salonen K, Bucher U, Nydegger UE. Comparison of post-transfusion recoveries achieved with either fresh or stored platelet concentrates. *Blut* 1987; 54: 207–212.
32 Norol F, Kuentz M, Cordonnier C et al. Influence of clinical status on the efficiency of stored platelet transfusion. *Br J Haematol* 1994; 86: 125–129.
33 Hogge DE, Thompson BW, Schiffer CA. Platelet storage for 7 days in second-generation blood bags. *Transfusion* 1986; 26: 131–135.
34 Klein HG, Dodd RY, Ness PM et al. Current status or microbial contamination of blood components: summary of a conference. *Transfusion* 1997; 37: 95–101.

Cambridge Books Online © Cambridge University Press, 2009

35 Lin L, Cook DN, Wiesehahn GP et al. Photochemical inactivation of viruses and bacteria in platelet concentrates by use of a novel psoralen and long-wavelength ultraviolet light. *Transfusion* 1997; 37: 423–435.
36 Mitchell KM, Brecher ME. Approaches to the detection of bacterial contamination in cellular blood products. *Transfus Med Rev* 1999; 13: 132–144.
37 Metcalfe P, Williamson LM, Reutelingsperger CPM et al. Activation during preparation of therapeutic platelets affects deterioration during storage: a comparative flow cytometric study of different production methods. *Br J Haemat* 1997; 98: 86–95.
38 Li J, Xia Y, Bertino AM et al. The mechanism of apoptosis in human platelets during storage. *Transfusion* 2000; 40: 1320–1329.
39 Shapira S, Friedman Z, Shapiro H et al. The effect of storage on the expression of platelet membrane phosphatidylserine and the subsequent impact on the coagulant function of stored platelets. *Transfusion* 2000; 40: 1257–1263.
40 Holme S, Moroff G, Murphy S. A multi-laboratory evaluation of in vitro platelet assays: the tests for extent of shape change and response to hypotonic shock. *Transfusion* 1998; 38: 31–40.
41 Bertolini F, Murphy S. A multicenter evaluation of reproducibility of swirling in platelet concentrates. *Transfusion* 1994; 34: 796–801.
42 Bertolini F. The absence of swirling in platelet concentrates is highly predictive of poor posttransfusion platelet count increments and increased risk of a transfusion reaction. *Transfusion* 2000; 40: 121–122.
43 Murphy S, Sayar SN, Abdou NL et al. Platelet preservation by freezing. Use of dimethylsulfoxide as cryoprotective agent. *Transfusion* 1975; 14: 139–144.
44 Barnard MR, MacGregor H, Ragno G et al. Fresh, liquid-preserved, cryopreserved platelets: adhesive surface receptors and membrane procoagulant activity. *Transfusion* 1999; 39: 880–888.
45 Towell BL, Levine SP, Knight WA III et al. A comparison of frozen and fresh platelet concentrates in the support of thrombocytopenic patients. *Transfusion* 1986; 26: 525–530.
46 Schiffer CA, Aisner J, Wiernik PH. Frozen autologous platelet transfusion for patients with leukemia. *N Engl J Med* 1978; 299: 7–12.
47 Pedrazzoli P, Noris P, Perotti C et al. Transfusion of platelet concentrates cryopreserved with ThromboSol plus low-dose dimethylsulphoxide in patients with severe thrombocytopenia: a pilot study. *Br J Haemat* 2000; 108: 653–659.
48 Lee DH, Blajchman MA. Novel platelet products and substitutes. *Transfus Med Rev* 1998; 12: 175–187.
49 Mannucci PM. Hemostatic drugs. *N Engl J Med* 1998; 339: 245–253.
50 Aster RH. Pooling of platelets in the spleen: role in the pathogenesis of 'hypersplenic thrombocytopenia'. *J Clin Invest* 1966; 45: 645.
51 Bishop JF, McGrath K, Wolf MM et al. Clinical factors influencing the efficacy of pooled platelet transfusions. *Blood* 1988; 71: 383–387.
52 Davis KB, Slichter SJ, Corash L. Corrected count increment and percent platelet recovery as measures of posttransfusion platelet response: problems and a solution. *Transfusion* 1999; 39: 586–592.
53 O'Connell B, Lee EJ, Schiffer CA. The value of 10-minute posttransfusion platelet counts. *Transfusion* 1988; 28: 66–67.
54 Friedberg RC, Donnelly SF, Boyd JC et al. Clinical and blood bank factors in the management of platelet refractoriness and alloimmunization. *Blood* 1993; 81: 3428–3434.
55 Klumpp TR, Herman J, Innis S et al. Factors associated with response to platelet transfusion following hematopoietic stem cell transplantation. *Bone Marrow Transpl* 1996; 17: 1035–1041.
56 Doughty HA, Murphy MF, Metcalfe P et al. Relative importance of immune and non-immune causes of platelet refractoriness. *Vox Sang* 1994; 66: 200–205.
57 Alcorta I, Pereira A, Ordinas A. Clinical and laboratory factors associated with platelet transfusion refractoriness: a case-control study. *Br J Haematol* 1996; 93: 220–224.
58 Bock M, Muggenthaler KH, Schmidt U et al. Influence of antibiotics on posttransfusion platelet increment. *Transfusion* 1996; 36: 952–954.
59 Bishop JF, Matthews JP, McGrath K et al. Factors influencing 20-hour increments after platelet transfusion. *Transfusion* 1991; 31: 392–396.
60 Hanson SR, Slichter SJ. Platelet kinetics in patients with bone marrow hypoplasia: evidence for a fixed platelet requirement. *Blood* 1985; 66: 1105–1109.
61 Hersh JK, Hom EG, Brecher ME. Mathematical modeling of platelet survival with implications of optimal transfusion practice in the chronically platelet transfusion-dependent patient. *Transfusion* 1998; 38: 637–644.
62 Norol F, Bierling P, Roudot-Thoraval F et al. Platelet transfusion: a dose-response study. *Blood* 1998; 92: 1448–1453.
63 Klumpp TR, Herman JH, Gaughan JP et al. Clinical consequences of alterations in platelet transfusion dose: a prospective, randomized, double-blind trial. *Transfusion* 1999; 39: 674–681.
64 McVay PA, Toy PT. Lack of increased bleeding after liver biopsy in patients with mild hemostatic abnormalities. *Am J Clin Pathol* 1990; 94: 747–753.
65 Brecher ME. Letter to the editor concerning: Klumpp et al. Clinical consequences of alterations in platelet transfusion dose: a prospective, randomized, double-blind trial. *Transfusion* 2000; 40: 383–384.
66 Gaydos LA, Freireich EJ, Mantel N. The quantitative relation between platelet count and hemorrhage in patients with acute leukemia. *N Engl J Med* 1962; 266: 905–909.
67 Freireich EJ, Kliman A, Lawrence AG, Mantel N, Frei E. Response to repeated platelet transfusion from the same donor. *Ann Intern Med* 1963; 59: 277–287.
68 Gmur J, Burger J, Schanz U et al. Safety of stringent prophylactic platelet transfusion policy for patients with acute leukemia. *Lancet* 1991; 338: 1223–1226.

Cambridge Books Online © Cambridge University Press, 2009

69 Sagmeister M, Oec L, Gmur J. A restrictive platelet transfusion policy allowing long-term support of outpatients with severe aplastic anemia. *Blood* 1999; 93: 3124–3126.
70 Heckman KD, Weiner GJ, Davis CS et al. Randomized study of prophylactic platelet transfusion threshold during induction therapy for adult acute leukemia: 10,000/μL versus 20,000/μL. *J Clin Oncol* 1997; 15: 1143–1149.
71 Rebulla P, Finazzi G, Marangoni F et al. The threshold for prophylactic platelet transfusions in adults with acute myeloid leukemia. *N Engl J Med* 1997; 337: 1870–1875.
72 Wandt H, Frank M, Ehninger G et al. Safety and cost effectiveness of a 10×10^9/L trigger for prophylactic platelet transfusion compared with the traditional 20×10^9/L trigger: A prospective comparative trial in 105 patients with acute myeloid leukemia. *Blood* 1998; 91: 3601–3606.
73 Contreras M. The appropriate use of platelets: An update from the Edinburgh Consensus Conference. *Br J Haemat* 1998; 101: 10–12.
74 Nevo S, Swan V, Enger C et al. Acute bleeding after bone marrow transplantation (BMT) – incidence and effect on survival. A quantitative analysis in 1,402 patients. *Blood* 1998; 91: 1469–1477.
75 Bernstein SH, Nademanee AP, Vose JM et al. A multicenter study of platelet recovery and utilization in patients after myeloablative therapy and hematopoietic stem cell transplantation. *Blood* 1998; 91: 3509–3517.
76 Reed RL, Ciavarella D, Heimbach DM et al. Prophylactic platelet administration during massive transfusion. A prospective, randomized, double-blind clinical study. *Ann Surg* 1986; 203: 40–48.
77 Simon TL, Aki Bechara F, Murphy W. Controlled trial of routine administration of platelet concentrates in cardiopulmonary bypass surgery. *Ann Thorac Surg* 1984; 37: 359–364.
78 Carr JM, Kruskall MS, Kaye JA et al. Efficacy of platelet transfusions in immune thrombocytopenia. *Am J Med* 1986; 80: 1051–1054.
79 McIntoch S, O'Brien RT, Schwartz AD et al. Neonatal isoimmune purpura: response to platelet infusions. *J Pediatr* 1973; 82: 1020–1027.
80 Win N. Provision of random-donor platelets (HPA-1a positive) in neonatal alloimmune thrombocytopenia due to anti HPA-1a alloantibodies. *Vox Sang* 1996; 71: 130–131.
81 Munizza M, Nance S, Keashen-Schnell MA, Sherwood W, Murphy S. Provision of HPA-1a (PL^{A1}) negative platelets for neonatal alloimmune thrombocytopenia: screening, testing and transfusion protocol. *Immunohematology* 1999; 15: 71–74.
82 Taening E. HLA antibodies and fetomaternal alloimmune thrombocytopenia: myth or meaningful? *Transfus Med Rev* 2000; 14: 275–280.
83 Kaplan C, Daffos F, Forestier F et al. Management of alloimmune thrombocytopenia: antenatal diagnosis and in utero transfusion of maternal platelets. *Blood* 1988; 72: 340–343.
84 Dumont LJ, Krailadsiri P, Seghatchian J et al. Preparation and storage characteristics of white cell-reduced high-concentration platelet concentrates collected by an apheresis system for transfusions in utero. *Transfusion* 2000; 40: 91–100.
85 Win N, Matthey F, Slater NG. Blood components – transfusion support in post-transfusion purpura due to HPA-1a immunization. *Vox Sang* 1996; 71: 191–193.
86 Castillo R, Monteagudo J, Escolar G et al. Hemostatic effect of normal platelet transfusion in severe von Willebrand disease individuals. *Blood* 1991; 77: 1901–1905.
87 Bell WR, Braine HG, Ness PM, Kickler TS. Improved survival in thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. *N Engl J Med* 1991; 325: 398–403.
88 Warkentin TE, Kelton JG. Heparin and platelets. *Hemat/Oncol Clinics of N Amer* 1990; 4: 243–264.
89 Kao KJ, del Rosario MLU. Role of class-II major histocompatibility complex (MHC)-antigen-positive donor leukocytes in transfusion-induced alloimmunization to donor class-I MHC antigens. *Blood* 1998; 92: 690–694.
90 The trial to reduce alloimmunization to platelets study group. Leukocyte reduction and ultraviolet B irradiation of platelets to prevent alloimmunization and refractoriness to platelet transfusions. *N Engl J Med* 1997; 337: 1861–1869.
91 Vamvakas EC. Meta-analysis of randomized controlled trials of the efficacy of white cell reduction in preventing HLA-alloimmunization and refractoriness to random-donor platelet transfusions. *Trans Med Rev* 1998; 12: 258–270.
92 Sintnicolaas K, van Marwijk Kooij M, Van Prooijen HC et al. Leukocyte depletion of random single-donor platelet transfusions does not prevent secondary human leukocyte antigen-alloimmunization and refractoriness: a randomized prospective study. *Blood* 1995; 85: 824–828.
93 Bishop JF, Matthews JP, Yuen K et al. The definition of refractoriness to platelet transfusions. *Transfus Med* 1992; 2: 35–41.
94 Hogge DE, Dutcher JP, Aisner J et al. Lymphocytotoxic antibody is a predictor of response to random donor platelet transfusion. *Am J Hematol* 1983; 14: 363–369.
95 McFarland JG, Anderson AJ, Slichter SJ. Factors influencing the transfusion response to HLA-selected apheresis donor platelets in patients refractory to random platelet concentrates. *Br J Haematol* 1989; 73: 380–386.
96 Phekoo KJ, Hambley H, Schey SA et al. Audit of practice in platelet refractoriness. *Vox Sang* 1997; 73: 81–86.
97 Engelfriet CP, Reesink HW. Management of alloimmunized, refractory patients in need of platelet transfusions. *Vox Sang* 1997; 73: 191–198.
98 Dutcher JP, Schiffer CA, Aisner J et al. Long-term follow-up of patients with leukemia receiving platelet transfusions: identification of a large group of patients who do not become alloimmunized. *Blood* 1981; 58: 1007–1011.
99 Dutcher JP, Schiffer CA, Aisner J et al. Alloimmunization following platelet transfusion: the absence of a dose-response relationship. *Blood* 1981; 57: 395–398.
100 Lee EJ, Schiffer CA. Serial measurement of lymphocytotoxic antibody and response to nonmatched platelet transfusion in alloimmunized patients. *Blood* 1987; 70: 1727–1729.
101 Petz LD, Garratty G, Calhoun L et al. Selecting donors of

Cambridge Books Online © Cambridge University Press, 2009

platelets for refractory patients on the basis of HLA antibody specificity. *Transfusion* 2000; 40: 1446–1456.
102 Murphy S, Varma M. Selecting platelets for transfusion of the alloimmunized patient: a review. *Immunohematology* 1998; 14: 117–123.
103 Rodey GE, Neylan JF, Whelchel JD, Revels KW, Bray RA. Epitope specificity of HLA Class I alloantibodies. I. Frequency analysis of antibodies to private versus public specificities in potential transplant recipients. *Hum Immunol* 1994; 39: 272–280.
104 Zimmermann R, Wittmann G, Zingsem J et al. Antibodies to private and public HLA class I epitopes in platelet recipients. *Transfusion* 1999; 39: 772–780.
105 MacPherson BR. HLA antibody formation within the HLA-A1 crossreactive group in multitransfused platelet recipients. *Am J Hematol* 1989; 30: 228–232.
106 Yankee RA, Grumet FC, Rogentine GN. Platelet transfusion. The selection of compatible platelet donors for refractory patients by lymphocyte HL-A typing. *N Engl J Med* 1969; 281: 1208–1212.
107 Duquesnoy RJ, Filip DJ, Rodey GE et al. Successful transfusion of platelets 'mismatched' for HLA antigens to alloimmunized thrombocytopenic patients. *Am J Hematol* 1977; 22: 219–226.
108 Dahlke MB, Weiss KL. Platelet transfusion from donors mismatched for crossreactive HLA antigens. *Transfusion* 1984; 24: 299–302.
109 Moroff G, Garratty G, Heal JM et al. Selection of platelets for refractory patients by HLA matching and prospective crossmatching. *Transfusion* 1992; 32: 633–40.
110 Gelb AB, Leavitt AD. Crossmatch-compatible platelets improve corrected count increments in patients who are refractory to randomly selected platelets. *Transfusion* 1997; 37: 624–630.
111 Santoso S, Kiefel V, Mueller-Eckhardt C. Blood group A and B determinants are expressed on platelet glycoproteins IIa, IIIa, Ib. *Thromb Haemost* 1991; 65: 196–201.
112 Lee EJ, Schiffer CA. ABO compatibility can influence the results of platelet transfusion. *Transfusion* 1989; 29: 384–389.
113 Brand A, Sintnicolaas K, Claas FJH et al. ABH antibodies causing platelet transfusion refractoriness. *Transfusion* 1986; 26: 463–466.
114 Mair B, Benson K. Evaluation of changes in hemoglobin levels associated with ABO-incompatible plasma in apheresis platelets. *Transfusion* 198; 38: 51–55.
115 McManigal S, Sims KL. Intravascular hemolysis secondary to ABO incompatible platelet products. An under-recognized transfusion reaction. *Am J Clin Pathol* 1999; 111: 202–206.
116 Larsson LG, Welsh VJ, Ladd DJ. Acute intravascular hemolysis secondary to out-of-group platelet transfusion. *Transfusion* 2000; 40: 902–906.
117 Heal JM, Blumberg N. The second century of ABO: and now for something completely different. *Transfusion* 1999; 39: 1155–1159.
118 Langenscheidt F, Kiefel V, Santoso S et al. Platelet transfusion refractoriness associated with two rare platelet-specific alloantibodies (anti-Bak^{a} and anti-P1^{A2}) and multiple HLA antibodies. *Transfusion* 1988; 28: 597–600.
119 Ikeda H, Mitani T, Ohnuma M et al. A new platelet-specific antigen, Nak^{a}, involved in the refractoriness of HLA-matched platelet transfusion. *Vox Sang* 1989; 57: 213–217.
120 Saji H, Maruya E, Fujii H et al. New platelet antigen, Siba, involved in platelet transfusion refractoriness in a Japanese man. *Vox Sang* 1989; 56: 283–287.
121 Kekomaki S, Volin L, Koistinen P et al. Successful treatment of platelet transfusion refractoriness: the use of platelet transfusions matched for both human leukocyte antigens (HLA) and human platelet alloantigens (HPA) in alloimmunized patients with leukemia. *Eur J Haematol* 1998; 60: 112–118.
122 Mangano MM, Chambers LA, Kruskall MS. Limited efficacy of leukopoor platelets for prevention of febrile transfusion reactions. *Am J Clin Pathol* 1991; 95: 733–738.
123 Goodnough LT, Riddell J, Lazarus H et al. Prevalence of platelet transfusion reactions before and after implementation of leukocyte-depleted platelet concentrates by filtration. *Vox Sang* 1993; 65: 103–107.
124 Heddle NM, Klama L, Singer J et al. The role of the plasma from platelet concentrates in transfusion reactions. *N Engl J Med* 1994; 331: 625–671.
125 Heddle NM, Klama L, Meyer R et al. A randomized controlled trial comparing plasma removal with white cell reduction to prevent reactions to platelets. *Transfusion* 1999; 39: 231–238.
126 Sarkodee-Adoo CB, Kendall JM, Sridhara R et al. The relationship between the duration of platelet storage and the development of transfusion reactions. *Transfusion* 1998; 38: 229–235.
127 Kelley DL, Mangini J, Lopez-Plaza I, Triulzi DJ. The utility of ≤3-day-old whole-blood platelets in reducing the incidence of febrile nonhemolytic transfusion reactions. *Transfusion* 2000; 40: 439–442.
128 Federowicz I, Barrett BB, Anderson JW et al. Characterization of reactions after transfusion of cellular blood components that are white cell reduced before storage. *Transfusion* 1996; 36: 21–28.
129 Kluter H, Bubel S, Kirchner H, Wilhelm D. Febrile and allergic transfusion reactions after the transfusion of white cell-poor platelet preparations. *Transfusion* 1999; 39: 1179–1184.
130 Buck SA, Kickler TS, McGuire M et al. The utility of platelet washing using an automated procedure for severe platelet allergic reactions. *Transfusion* 1987; 27: 391–393.
131 Hume HA, Popovsky MA, Benson K et al. Hypotensive reactions: a previously uncharacterized complication of platelet transfusion? *Transfusion* 1996; 36: 904–909.
132 Cyr M, Hume HA, Champagne M et al. Anomaly of the des-Arg^{9}-bradykinin metabolism associated with severe hypotensive reactions during blood transfusions: a preliminary study. *Transfusion* 1999; 39: 1084–1088.
133 Bowden RA, Slichter SJ, Sayers M et al. A comparison of filtered leukocyte-reduced and cytomegalovirus (CMV) seronegative blood products for the prevention of transfu-

Cambridge Books Online © Cambridge University Press, 2009

sion-associated CMV infection after marrow transplant. *Blood* 1995; 86: 3598–3603.
134 Leitman SF, Holland PV. Irradiation of blood products. Indications and guidelines. *Transfusion* 1985; 25: 293–303.
135 Goldfinger D, McGinniss MH. Rh-incompatible platelet transfusion – risks and consequences of sensitizing immunosuppressed patients. *N Engl J Med* 1971; 284: 942–944.
136 AuBuchon JP, Kruskall MS. Transfusion safety: realigning efforts and risks. *Transfusion* 1997; 37: 1211–1216.
137 Svoboda R, Lipton KS. Bacterial contamination of blood components. *AABB Assoc Bull* 1996; 96(6): 2–4.
138 Grass JA, Hei DJ, Metchette K et al. Inactivation of leukocytes in platelet concentrates by photochemical treatment with psoralen plus UVA. *Blood* 1998; 91: 2180–2188.
139 Ramanathan RK, Triulzi DJ, Logan TF. Transfusion-related acute lung injury following random donor platelet transfusion: a report of two cases. *Vox Sang* 1997; 73: 43–45.

Cambridge Books Online © Cambridge University Press, 2009

Cambridge Books Online © Cambridge University Press, 2009

Part III

B Thrombosis

Cambridge Books Online © Cambridge University Press, 2009

Cambridge Books Online © Cambridge University Press, 2009

47

Pathophysiology of arterial thrombosis

Lina Badimon[1], Juan-Jose Badimon[2], and Valentin Fuster[2]

[1]*Cardiovascular Research Center, CSIC-HSCSP-UAB, Barcelona, Spain*
[2]*Cardiovascular Institute, Mount Sinai School of Medicine, New York, USA*

During the past few years we have witnessed a remarkable advance in our understanding of the pathophysiology of coronary atherosclerosis–thrombosis. Here we focus on thrombosis with specific emphasis on the coronary arteries[1–4].

Lesion classification and progression of atherosclerosis–thrombosis

According to the criteria of the American Heart Association Committee on Vascular lesions, plaque progression can be divided into the five phases and various lesion types shown in Fig 47.1[5–7]. The so-called 'vulnerable' type IV and type Va lesions (phase 2) and the so-called 'complicated' type VI lesion (phase 4) are the most relevant to acute coronary syndromes (ACS). The acute type VI lesion that results in an ACS, rather than being characterized by a small mural thrombus, consists of an occlusive thrombus.

Type IV and type Va lesions, although not necessarily stenotic at angiography, may be prone to disruption because of their softness due to a high lipid content and macrophage-dependent chemical properties. Type IV lesions consist of confluent cellular lesions with a great deal of extracellular lipid intermixed with fibrous tissue covered by a fibrous cap, whereas type Va lesions possess a predominant extracellular lipid core also covered by a thin fibrous cap. Phase 2 can evolve into acute phase 3 or 4, and either of them can evolve into a fibrotic phase 5.

Vulnerable lipid-rich plaque and its disruption

Type IV and type Va plaques are commonly composed of an abundant crescentic mass of lipids, separated from the vessel lumen by a discrete component of extracellular matrix (Figs. 47.1, 47.2). Fairly small coronary lesions by angiography may be associated with acute progression to severe stenosis or total occlusion and may eventually account for as many as two-thirds of the patients in whom unstable angina or other ACS develop[8–10]. This unpredictable and episodic progression is most probably caused by disruption of type IV and type V plaques with subsequent thrombus formation, which changes the plaque geometry and leads to acute, or intermittent plaque growth and acute occlusive coronary syndromes. Such plaque disruption seems to depend on both passive and active phenomena.

Passive disruption of plaques

Related to physical forces, passive plaque disruption occurs most frequently where the fibrous cap is thinnest, most heavily infiltrated by foam cells, and therefore weakest. For eccentric plaques, this is often the shoulder or between the plaque and the adjacent vessel wall[11]. Pathoanatomical examination of intact and disrupted plaques and in vitro mechanical testing of isolated fibrous caps from aorta indicate that vulnerability to rupture depends on three factors: circumferential wall stress or cap 'fatigue'; location, size and consistency of the atheromatous core; and blood-flow characteristics, particularly the impact of flow on the proximal aspect of the plaque (i.e. configuration and angulations of the plaque)[5,8,11].

Active disruption plaques

The process of plaque disruption is not purely mechanical. Atherectomy specimens from patients with ACS reveal areas very rich in macrophages[12], and these cells are

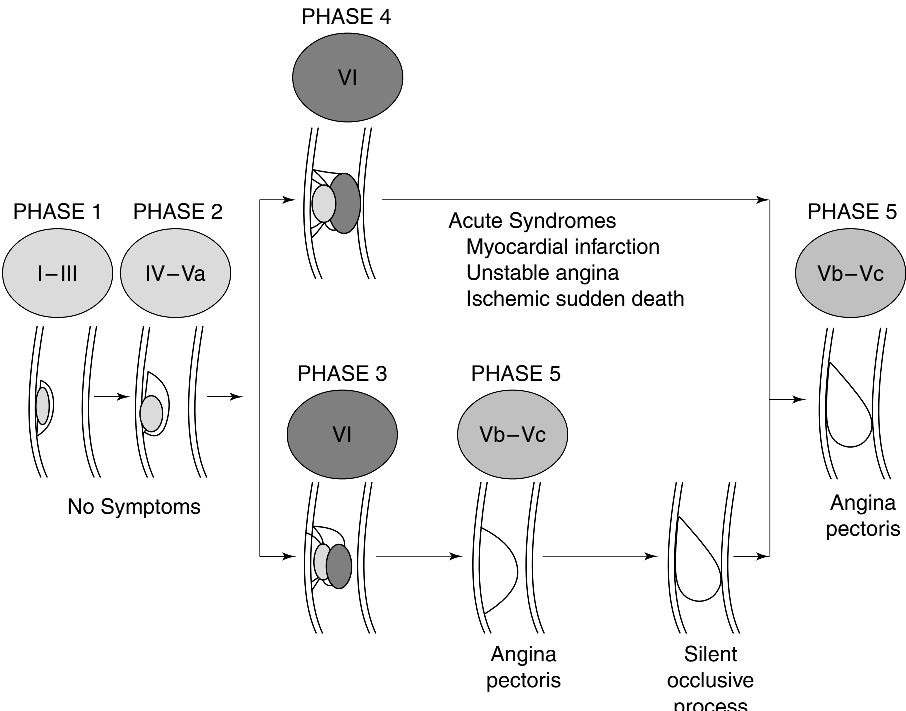


Fig. 47.1. Phases and lesion morphology of progression of coronary atherosclerosis according to gross pathological and clinical findings.

capable of degrading extracellular matrix by phagocytosis or secretion of proteolytic enzymes. Thus, enzymes such as plasminogen activators and matrix metalloproteinases (MMPs, collagenases, gelatinases, and stromelysins) may weaken the fibrous cap and predispose it to rupture[13].

Acute thrombosis

Disruption of a vulnerable or unstable plaque with a subsequent change in plaque geometry and thrombosis results in complicated lesions (Figs. 47.1 and 47.2). Such a rapid change in the geometry of atherosclerotic plaques may result in acute occlusion or subocclusion with clinical manifestations of unstable angina or other ACS. More frequently, however, the rapid changes seem to result in mural thrombus without evident clinical symptoms, which, by self-organization, may be a main contributor to the progression of atherosclerosis. More specifically, at the time of coronary plaque disruption, a number of local and systematic thrombogenic factors may influence the degree and the duration of thrombus deposition (Table 47.1). Such a thrombus may then either by partially lysed or become replaced in the process of organization by the vascular repair response.

Substrate and platelet dependent thrombosis

The platelet is a fundamental element in thrombosis[14]. In the areas with vascular lesion exposure of collagen to circulating blood and thrombin generated through the activation of the coagulation cascade, in addition to systemic agonists, can cooperate to produce a potent and rapid platelet activation process. Another pathway for platelet activation is mediated through ADP that is released by hemolysis of red blood cells in the areas of vascular lesion. ADP stimulates calcium discharge and the release of the platelet granular content. ADP produces the stimulation of neighbouring platelets, recruitment and the triggering of the full reaction for platelet aggregation and thrombus formation.

Serotonin, vasoconstrictor factor derived from the platelet can also stimulate the adrenergic nervous system in the blood vessels. Serotonin receptors are present in the coronary arteries and their stimulation produces vasoconstriction[15]. Dense granules in the platelets are also the place of storage of ADP and calcium. In addition, adhesive proteins such as fibrinogen, fibronectin, vWF, thrombospondin, and vitronectin are stored in the alpha granules of the platelets. The alpha granules contain proteins that promote growth, such as PDGF, TGFβ, and platelet factor

Cambridge Books Online © Cambridge University Press, 2009

Fig. 47.2. Plaque vulnerability, disruption and thrombosis: anatomical changes leading to ACS and subsequent plaque remodelling.

4[16–18]. The alpha granules are also the place of storage of coagulation factors such as factor V, high molecular weight kininogen (HMWK), factor XI, protein S and PAI 1. In these granules albumin and immunoglobulins are also stored. The components of alpha granules are released by the platelets at a lower concentration of agonist than those needed to release the constituents of the dense granules[19,20].

Any of the possible mechanisms of platelet activation induce the subsequent exposure of the platelet receptor glycoproteins (GP) IIb/IIIa. Adhesive molecules such as fibrinogen, vWF, and fibronectin can bind to GP IIb/IIIa and form bridges with other platelets triggering the complete process of platelet aggregation (Fig. 47.3).

In the interaction of the platelet with the vascular wall two surface glycoprotein receptors play a fundamental role. The GP Ib/IX complex is needed for the normal adhesion of the platelets to subendothelial areas, mainly at high shear rate conditions[21,22] presumably through its interaction with vWF[23]. vWF also can bind GP IIb/IIIa[24]. Several studies have suggested a double role for these glycoproteins; thus, they have a role in adhesion and also in platelet to platelet interactions that lead to thrombus formation[22–28]. We may thus conclude that in conditions of high shear rate GP Ib and GP IIb/IIIa are involved in the process of platelet adhesion while GP IIb/IIIa are involved mainly in platelet to platelet interaction. Platelet activation induces structural changes in GP IIb/IIIa that lead to the formation of a heterodimeric complex that works as the receptor for fibrinogen. ADP induces further exposition of binding sites for fibrinogen on the platelet surface. This process generates cohesive binding between platelets and

Cambridge Books Online © Cambridge University Press, 2009

Table 47.1. Thrombotic complications of plaque disruption: local and systemic thrombogenic risk factors

Local factors
Degree of plaque disruption (i.e. erosion, ulcer)
Degree of stenosis (i.e. change in geometry)
Tissue substrate (i.e. lipid-rich plaque)
Surface of residual thrombus (i.e. recurrence)
Vasoconstriction (i.e. platelets, thrombin)

Systemic factors
Cholesterol, Lp(a)
Catecholamines (i.e. smoking, stress, cocaine)
Fibrinogen, impaired fibrinolysis (i.e. PAI-1), activated platelets and clottings (i.e. factor VII, thrombin generation, factor 112, or activity-FPA)
Infections (*Chlamydia pneumoniae*, cytomegalovirus, *Helicobacter pylori*)?

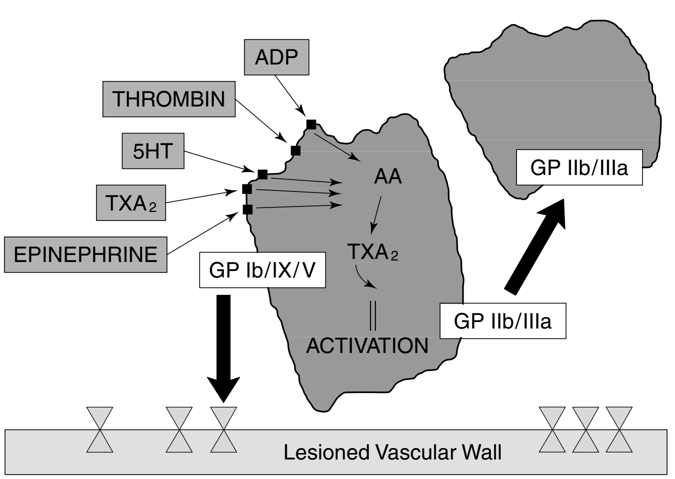


Fig. 47.3. Diagram of platelet activation, adhesion and aggregation.

therefore the growth of the thrombus. Fibrinogen molecules, with dimeric structure, form bridges between GP IIb/IIIa molecules in neighbouring platelets.

New platelet recruitment due to the platelet released products are probably the result of the action of ADP and thromboxane A_2. It is important to emphasize that recruitment produces a significant activation, potentiating aggregation and increasing the thrombotic process.

Finally the thrombotic mass will be consolidated because of the formation of thrombin that can induce the total occlusion of the vessel. The process of thrombus growth, that initially was part of a hemostatic response, will be transformed into an occlusive thrombus that will interrupt blood flow. The intact red blood cells that are reaching the area of thrombus growth respond to the released platelet secretion products increasing the production of prothrombotic substances including arachidonate and eicosanoids[29,30]. This phase of secondary consolidation of the thrombus, because of thrombin, completes the thrombotic process.

Table 47.2. Platelet receptors

Ligand	Receptor	Other names
Collagen	GPIa–IIa	VLA-2, $\alpha_2\beta_1$
	GPIIb–IIIa	$\alpha IIb\beta_3$
	GPIV	GPIIIb
Fibrinogen	GPIIb–IIIa	$aIIbb_3$
Fibronectin	GPIc–IIa	VLA-5, $\alpha_5\beta_1$
	GPIIb–IIIa	$\alpha IIb\beta_3$
Thrombospondin	Vn-R	$\alpha_v\beta_3$
	GPIV	GPIIIb
Vitronectin	Vn-R	$\alpha_v\beta_3$
	GPIIb–IIIa	$\alpha IIb\beta_3$
vWF	GPIb–IX	–
	GPIIb–IIIa	$\alpha IIb\beta_3$
Laminin	GPIc–IIa region	VLA-6, a_6b_1

Note:
Vn-R, Vitronectin receptor; vWF, von Willebrand factor.

Glycoproteins and the platelets membrane

Platelet function depends on adhesive interactions and many of the surface glycoproteins in the platelet membrane are receptors for adhesive proteins (Table 47.2). Some of these receptors have been identified, cloned, sequenced and classified among large families of genes that mediate diverse types of cellular interactions[31]. The most abundant is the family of the integrins that includes GP IIb/IIIa, GPIa/IIa, GP Ic/IIa and the receptors of fibronectin and vitronectin in decreasing order of magnitude. The family of genes that include the leucine-rich glycoproteins is represented by the complex of GPIb, a receptor for vWF in unstimulated platelets that is involved in the binding subendothelium, and GP V. Other families of genes include the selectins (GMP 140) and PECAM-1. In addition there is a family of genes that include GP IV[31].

Cambridge Books Online © Cambridge University Press, 2009

Glycoprotein Ib (GPIb)

The GPIb complex is the glycoprotein responsible for the negative charge of the surface of the platelets, due to its high content in sialic acid and carbohydrates. It has two disulfide linked subunits, GP Ibα (143 KDa) and GP Ibβ (22KDa), that are bound not covalently to GP IX in a heterodimeric one to one complex. There are approximately 25 000 molecules of GP Ib per platelet. GP Ibβ and GP IX are transmembrane glycoproteins and they form the globular domain of larger size. The elongated part of the receptor corresponds to the GP Ibα subunit. This glycoprotein complex links immobilized vWF exposed in the vascular subendothelium and initiates platelet adhesion. GP Ib does not bind to circulating plasma vWF because it requires a conformational change that is produced when it is bound to extracellular matrix to be recognized by the receptor. The vWF binding domain is located in aa 251–279 of the GP Ibα subunit[32]. The binding domain in vWF corresponds to a trypsin fragment that extends from aa 449 to 728 and it does not contain the sequence RGD[33]. The cytoplasmic domain of the receptor has as its main function the stabilization of the plasma membrane and maintenance of the platelet shape by linking the plasma membrane to intracellular actin filaments of the cytoskeleton.

Glycoproteins IIb/IIIa (GP IIb/IIIa)

On the surface of unstimulated platelets there are around 50 000 to 80 000 molecules of GP IIb/IIIa (integrin α IIb β_3). The complex is a heterodimer consisting of two transmembrane glycoproteins GP IIb of 136 kD (α-subunit) (GP IIb-β and GP IIb-α linked by disulfide bridges) and one of GP IIIa of 92 kD (β-subunit). In the human species GP IIb and IIIa are synthesized as precursors by different genes, both of them located in the region q21–22 of the larger arm of chromosome 17[34]. The transcription of the gene is also controlled by independent factors. The receptor IIb/IIIa is a calcium-dependent heterodimer that is linked in a noncovalent fashion[35]. Calcium is fundamental for the maintenance of the complex and for the binding of adhesive glycoproteins[36,37]. In activated platelets GP IIb/IIIa is a receptor for fibrinogen, fibronectin, vWF, vitronectin and thrombospondin[38]. The sequences to which the receptor is linked correspond to small peptide sequences RGD present in the adhesive proteins[39]. Fibrinogen contains two RGD sequences in the alpha chain, one in the N-terminal region (aa 95 to 97) and the second in the C-terminal (aa 572–574)[40]. Fibrinogen has a third recognition domain that corresponds to the sequences of 12 aa localized in the C-terminal of the gamma chain of the molecule. This dodecapeptide is specific to fibrinogen and it does not contain the sequence RGD[41].

Activation and exposure of the GP IIb/IIIa receptors

In the non activated platelets the GP IIb–IIIa receptors are unable to bind plasma fibrinogen[42,43]. The receptor activity is acquired as a consequence of platelet activation because of the conformational changes in the heterodimer. Indeed, except for megakaryocytes and platelets this receptor cannot be activated when it is expressed in cell lines, indicating that the expression of this receptor is regulated by specific cell elements either in the lipidic micro-environment or by other intracellular factors[44,45]. Platelet activation signalling is transmitted by mechanisms not yet fully understood through the plasma membrane and produces changes in the relative orientation of the extracellular domain of the GP IIb/IIIa receptor[46]. The binding of plasma fibrinogen to the activated receptor produces changes in the conformation, both in the receptor and in the ligand. The link of GP IIb–IIIa/fibrinogen complexes to the platelet cytoskeleton not only facilitates the anchoring needed for clot retraction but also regulates the reorganization of the cytoskeleton and the formation of a macromolecular complex of enzymes and proteins involved in signal transduction mechanisms[46,47].

Mechanisms of signal transduction in the platelet

The binding of the agonists to the membrane receptors exposed on the platelet surface activates secondary messengers that include inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DG). IP3 produces calcium release from the dense tubular system, increasing free calcium concentration in the cytosol. DG activates a serine/threonine kinase, protein kinase (PK C), inducing its translocation to the membrane and the phosphorylation of proteins in serine and threonine residues. These changes induce granular secretion and exposure of the fibrinogen receptor. Simultaneously, the increase in cytosolic free calcium facilitates the release of arachidonate by phospholipase A2 (PLA2) from the platelet membrane phospholipids. This process can occur both in the plasma membrane and in the membrane of the dense tubular system. Arachidonate is metabolized to thromboxane A2 that is released from the cell, interacts with the receptors of the platelet surface and increases the activation of the platelet (Fig. 47.4).

Cambridge Books Online © Cambridge University Press, 2009

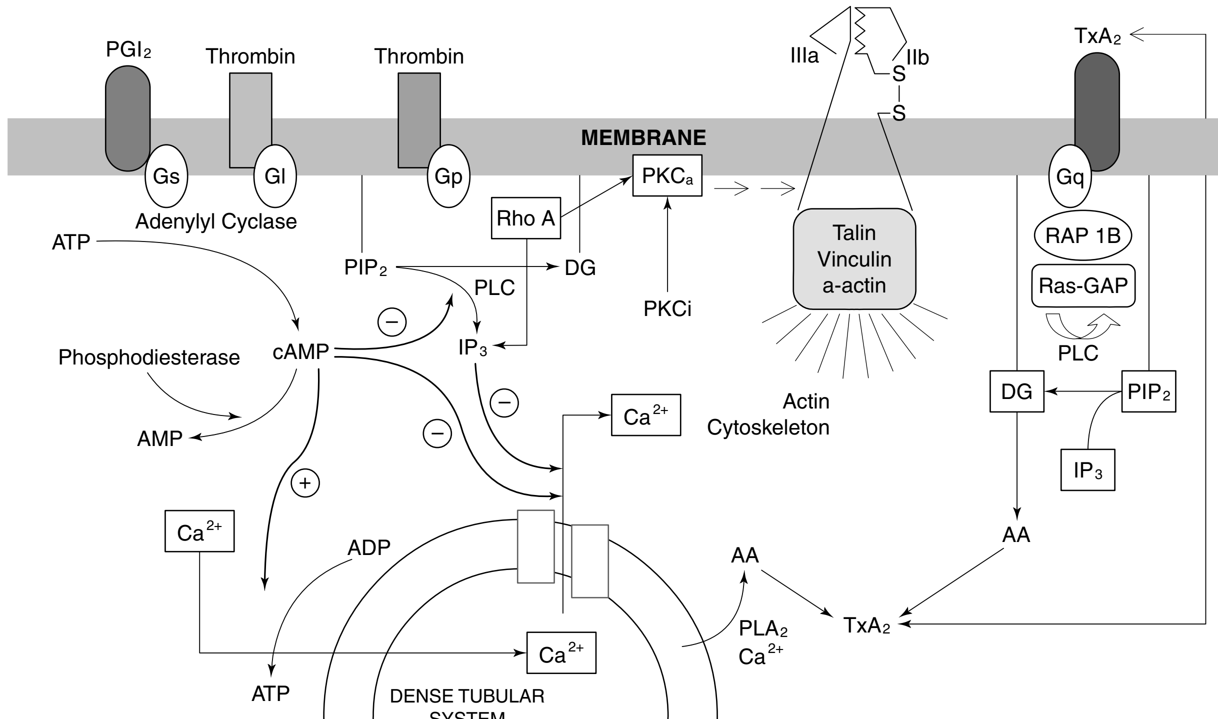


Fig. 47.4. Signal transduction mechanisms in platelet activation.

The activation of the platelet is inhibited when the levels of cyclic AMP (cAMP) are increased. This is the mechanism of action of some inhibitors of platelet function such as prostacyclin (PGI2). Similarly nitric oxide (NO) inhibits platelet function through the activation of guanil cyclase in the cytosol that increases the intracellular levels of cyclic GMP (cGMP)[48]. The elevation of cAMP levels produces the activation of cAMP-dependent protein kinases. Substrates for protein kinases are GP Ib (subunit beta), the actin binding protein, myosin light chain and Rap 1b. It is not fully known how these substrates promote the inhibition of platelet activation. In general agonists for platelet activation inhibit cAMP formation by blocking adenylate cyclase. Other compounds accelerate the metabolism of cAMP by the activation of the phosphodiesterase[49]. When cAMP levels increase in the platelet, free calcium is sequestered in the dense tubular system and PLA2 activity is inhibited[38].

Interaction between agonists and the enzymes responsible for the generation of second messengers is also mediated by G proteins. G proteins are heterotrimers formed by the subunits alpha, beta and gamma. The activation of G proteins is produced by binding to GTP (sometimes also to GDP). Platelets have at least nine types of G proteins. G proteins regulate the hydrolysis of phosphatidylcyoline and the formation of cAMP and are probably involved in the activation of PLA2 and adenylate cyclase[50,51]. Phospholipase C (PLC) is also activated by different G-proteins (Gp and Gq). Adenylate cyclase is stimulated by Gs and inhibited by Gi. We have very little information on PLA2 regulation by G-proteins. RAP1b, a G-protein of low molecular weight, has been shown to form a complex with PLC and Ras-GAP. Other low molecular weight G-proteins could be important in the regulation of vesicular transport and granular secretion in the platelet[52].

Substrate and tissue factor (TF/thrombin)

There is striking heterogeneity in the composition of human atherosclerotic plaques, even in the same individual, and the disruption of plaques exposes different vessel-wall components to blood. Data on the thrombogenicity of disrupted atherosclerotic lesions are limited. In two different experiments, human aortic plaques were exposed to flowing blood at high shear rate and their thrombogenicity was assessed. The studied material included normal intima (free of disease), fatty streaks, sclerotic plaques, fibrolipid plaques, and atheromatous lipid-rich core. The lipid core, abundant in cholesterol ester, displayed by far

Cambridge Books Online © Cambridge University Press, 2009

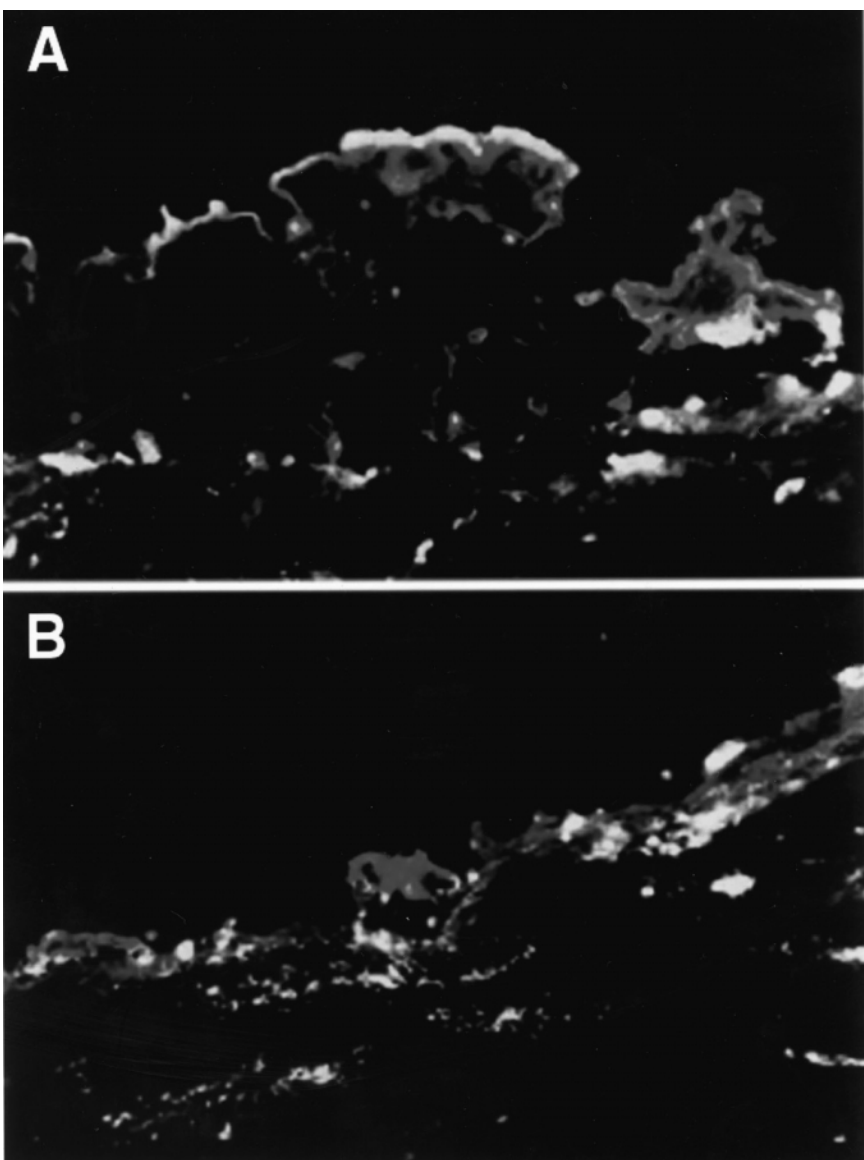


Fig. 47.5 (see also colour plate). Effects of TFPI on thrombogenicity of disrupted lipid-rich human atherosclerotic lesions: Control (*a*) and TFPI-treated (*b*). Fibrinogen deposition is shown as green, platelet deposition as red, and their colocalization as orange. Note the significant antithrombotic effect of rTFPI on deposition of platelet and fibrin(ogen) deposition.

the highest thrombogenicity and the most intense TF staining compared with other components[53,54].

TF, a small-molecular-weight glycoprotein, initiates the extrinsic clotting cascade and is believed to be a major regulator of coagulation, hemostasis, and thrombosis. TF forms a high-affinity complex that activates factors IX and X, which in turn leads to thrombin generation[55]. Colocalization analysis of coronary atherectomy specimens (culprit lesions) from patients with unstable angina showed a strong relation between TF and macrophages[56]. This relation suggests a cell-mediated thrombogenicity in patients with unstable angina and ACS. Furthermore, based on recent experimental observations, specific inhibition of vascular tissue factor by the use of r-tissue factor pathway inhibitor (rTFPi) was associated with a significant reduction of acute thrombus formation in lipid-rich plaques[57] (Fig. 47.5, see colour plate). Such observations document the role of TF activity in acute arterial thrombosis after atherosclerotic plaque disruption and open a new therapeutic strategy in the prevention of ACS.

Cambridge Books Online © Cambridge University Press, 2009

Hypercoagulable-dependent thrombosis

In regard to the thrombin generated by the primary activation of TF, the thrombin receptor has 425 aa with transmembrane domains and an NH_2-terminal extracellular domain that is cleaved by thrombin to produce a tethered ligand that binds and initiates the activation of the receptor and the signal transduction mechanisms[58]. Thrombin is a key enzyme in the formation of the thrombus, cleaving fibropeptides A and B from fibrinogen to form insoluble fibrin, which stabilizes the growing thrombus. Both free thrombin and thrombin bound to fibrin are able to transform fibrinogen into fibrin favouring the propagation of the thrombus in the area of the lesion. Simultaneously, platelet activation produces the expression of the fibrinogen receptor in the platelet membrane as discussed before. The process is perpetuated with the arrival of new platelets to the area of the lesion. There is evolving evidence that circulation monocytes and white blood cells may be involved in TF expression and thrombogenicity[59]; indeed, the predictive value for coronary events of high titres of C-reactive protein may be a manifestation of such systemic phenomena[60–63]. Hypercholesterolemia, a high catecholamine drive such as in cigarette smoking, certain chemotactic determinants, and perhaps infections may trigger such hypercoagulable phenomena (Table 47.1). Of interest, in about a third of ACS, particularly in acute sudden coronary death, there is no disruption of a fairly small lipid-rich plaque but just a superficial erosion of a markedly stenotic and fibrotic plaque[64,65]. Thus, complicated thrombi in such cases may well be dependent on a hypercoagulable state triggered by systemic factors mentioned above.

We have reported that normalization of plasma cholesterol concentrations by statins reduces the increased blood thrombogenicity observed in the same patients under hyperlipidemic conditions[66,67]. In addition, the mechanism of action was related more to the lipid-lowering activity than to the specific hypolipidaemic agent used. Systemic infections (e.g. with *Chlamydia pneumoniae*, cytomegalovirus, and *Helicobacter pylori*) have been linked to atherosclerotic disease and its thrombotic complications[68]; increased antibody titres predict future adverse events in patients with postmyocardial infarction. Infectious agents may activate circulating monocytes and white blood cells and create a hypercoagulable state (through synthesis and activation of TF and platelet interaction, as well as by raising fibrinogen concentration); however, recent antibiotic trials of the use of macrolide inhibitors have been inconclusive.

Thrombosis-related vasoconstriction

Although many episodes of unstable angina and acute myocardial infarction are caused by the disruption or erosion of a plaque with superimposed thrombosis, other mechanisms that alter myocardial oxygen supply and demand must be considered. Original studies by Maseri and colleagues[69] indicated that coronary vasoconstriction has an important role. In ACS, vasoconstriction may occur as a response to a mildly dysfunctional endothelium near the culprit lesion or, more likely, may be a response to deep arterial damage or plaque disruption of the culprit lesion itself. Thus, with regard to this second type of vasoconstriction, it seems that a predisposition exists for platelet-dependent and thrombin-dependent vasoconstriction at the site of plaque disruption and thrombosis that may be significant but transient[70]. Thus, platelet-dependent vasoconstriction, mediated by serotonin and thromboxane A_2[71] and thrombin-dependent vasoconstriction occur if the vascular wall has been damaged substantially with de-endothelialization, which suggests the direct interaction of these substances with the vascular smooth muscle cells.

Atherosclerosis-thrombosis and clinical consequences

Stable angina (usually exertional) or stable silent ischemia (exertional or not) commonly result from increases in myocardial oxygen demand that outstrip the ability of stenosed coronary arteries to increase oxygen delivery.

With regard to the pathobiology of ACS[72], in unstable angina, a fairly small fissuring of a lipid-rich plaque, and occasionally a superficial erosion of a fibrotic plaque, may lead to an acute change in plaque structure and a reduction in coronary blood flow, resulting in exacerbation of angina. Transient episodes of thrombotic-vessel occlusion at the site of plaque damage may occur, leading to angina at rest. This thrombus is usually labile and results in temporary vascular occlusion, perhaps lasting only 10–20 min. In addition, release of vasoactive substances by platelets (serotonin, thromboxane A_2), secondary to endothelial vasodilator dysfunction may alter perfusion and myocardial oxygen supply probably accounting for two-thirds of ACS, including episodes of unstable angina. The remainder one-third may be caused by transient increases in myocardial-oxygen demand.

In non-Q-wave myocardial infarction, more severe plaque damage results in more persistent thrombotic occlusion, perhaps lasting up to 1 h. Only about a fourth of patients with non-Q-wave myocardial infarction have an

Cambridge Books Online © Cambridge University Press, 2009

infarct-related vessel occluded for more than 1 h, but the distal myocardial territory is usually supplied by collaterals. Therefore, spontaneous thrombolysis, resolution of vasoconstriction, and presence of collateral circulation are important in preventing the formation of Q-wave myocardial ischemia. In Q-wave myocardial infarction, larger plaque fissures result in the formation of a fixed and persistent thrombus, which leads to an abrupt cessation of myocardial perfusion for more than 1 h, resulting in transmural necrosis of the involved myocardium. Some cases of sudden coronary death probably involve a rapidly progressive coronary lesion in which plaque disruption – commonly a superficial erosion – and resultant thrombosis lead to ischemic and fatal ventricular arrhythmias in the absence of collateral flow. Platelet microemboli may also contribute to the development of sudden ischemic death.

References

1 Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes. Parts I and II. *N Engl J Med* 1992; 326: 242–50, 310–388.

2 Davies MJ. Stability and instability: two faces of coronary atherosclerosis. *Circulation* 1996; 94: 2013–2020.

3 Steinberg D. Oxidative modification of LDL and atherogenesis. *Circulation* 1997; 95: 1062–1072.

4 Ross R. Atherosclerosis – an inflammatory disease. *N Engl J Med* 1999; 340: 115–126.

5 Fuster V. Mechanisms leading to myocardial infarction: insights from studies of vascular biology. *Circulation* 1994; 90; 2126–2146.

6 Stary HC, Stary HC, Chandler AB et al. A definition of advanced type of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation* 1995; 92: 1355–1374.

7 Fuster V, Gotto AM, Libby P, Loscalzo J, McGill HC. Matching the intensity of risk factor management with the hazard for coronary disease events. Pathogenesis of coronary disease: the biologic role of risk factors. *J Am Coll Cardiol* 1996; 27: 964–976.

8 Falk E, Shah PK, Fuster V. Coronary plaque disruption. *Circulation* 1995; 92: 657–671.

9 Ambrose JA, Tannenbaum MA, Alexopoulos D. Angiographic progression of coronary artery disease and the development of myocardial infarction. *J Am Coll Cardiol* 1988; 12: 56–62.

10 Little WC, Constantinescu M, Applegate RJ et al. Can coronary angiography predict the site of a subsequent myocardial infarction in patients with mild-to-moderate coronary artery disease? *Circulation* 1988; 78: 1157–1166.

11 Davies MJ, Richardson PD, Woolf N, Katz DR, Mann J. Risk of thrombosis in human atherosclerotic plaque: role of extracellular lipid, macrophages, and smooth muscle content. *Br Heart J* 1993; 69: 377–381.

12 Moreno PF, Falk E, Palacios IF, Newell JB, Fuster V, Fallon JT. Macrophage infiltration in acute coronary syndromes. Implications for plaque rupture. *Circulation* 1994; 90: 775–78.

13 Libby P. Molecular bases of the acute coronary syndromes. *Circulation* 1995; 91: 2844.

14 Badimon L, Badimon JJ, Fuster V. Pathogenesis of thrombosis. In Verstraete M, Fuster V, Topol EJ, eds. *Cardiovascular thrombosis*. Lippincott Raven Publishers; 1998: 23–44.

15 Flores NA, Sheridan DJ. The pathophysiological role of platelets during myocardial ischaemia. *Cardiovasc Res* 1994; 28: 295–302.

16 Rao AK. Congenital disorders of platelet function. *Hematol Oncol Clin N Am* 1990; 4: 65–86.

17 Zucker MB, Katz IR. Platelet factor 4: Production, structure, and physiologic and immunologic action. *Proc Soc Exp Biol Med* 1991; 198: 693–702.

18 Niewiarowski S. Secreted platelet proteins. In Bloom AL, Forbes CD, Thomas DP, Tuddenham EGD, eds. *Haemostasis and thrombosis*, 3rd edn. Vol. 1. Edinburgh: Churchill Livingstone;1994: 167–181.

19 Plow EF, Ginsberg MH. The molecular basis of platelet function. In Hoffman R, Benz EJ Jr, Shattil SJ, Furie B, Cohen HJ, eds. *Hematology, basic principles and practice*. New York: Churchill Livingstone; 1991: 1165–1176.

20 Holmsen H. Platelet secretion and energy metabolism. In Colman RW, Hirsh J, Marder VJ, Salzman EW, eds. *Hemostasis and thrombosis: basic principles and clinical practice*, 3rd edn. Philadelphia: Lippincott; 1994: 524–545.

21 Weiss HJ, Turitto VT, Baumgartner HR. Effect of shear rate on platelet interaction with subendothelium in citrated and native blood. Shear rate-dependent decrease of adhesion in von Willebrand's disease and the Bernard-Soulier syndrome. *J Lab Clin Med* 1978; 92: 750–754.

22 Sakariassen KS, Nievelstein PF, Coller BS, Sixma JJ. The role of platelet membrane glycoproteins Ib and IIb/IIIa in platelet adherence to human artery subendothelium. *Br J Haematol* 1986; 63: 681–691.

23 Sakariassen K, Bolhius PA, Sixma J. Human blood platelet adhesion to artery subendothelium is mediated by Factor VIII-von Willebrand factor bound to the subendothelium. *Nature* 1979; 636–638.

24 Badimon L, Badimonn JJ, Turitto VT, Fuster V. Platelet deposition in von Willebrand Factor deficient vessel wall. *J Lab Clin Med* 1987; 110: 634–647.

25 Badimon L, Badimon JJ, Turitto VT, Vallabhajosula S, Fuster V. Platelet thrombus formation on collagen type I. Influence of blood rheology, von Willebrand Factor and blood coagulation. *Circulation* 1988; 78: 1431–1442.

26 Badimon L, Badimon JJ, Chesebro JH, Fuster V. Inhibition of thrombus formation: blockage of adhesive glycoprotein mechanisms versus blockage of the cyclooxygenase pathway. *J Am Coll Cardiol* 1988; 11: 30A

27 Badimon L, Badimon JJ, Turitto VT, Fuster V. Platelet interaction to vessel wall and collagen. Study in homozygous von

Cambridge Books Online © Cambridge University Press, 2009

Willebrand's disease associated with abnormal collagen aggregation in swine. *Thromb Haemost* 1989; 61: 57–64.

28 Badimon L, Badimon JJ, Turitto VT, Fuster V. Role of von Willebrand factor in mediating platelet-vessel wall interaction at low shear rate; the importance of perfusion conditions. *Blood* 1989; 73: 961–967.

29 Santos MT, Valles J, Marcus AJ et al. Enhancement of platelet reactivity and modulation of eicosanoid production by intact erythrocytes. *J Clin Invest* 1991; 87: 571–580.

30 Valles J, Santos MT, Aznar J et al. Erythrocytes metabolically enhance collagen-induced platelet responsiveness via increased thromboxane production, ADP release, and recruitment. *Blood* 1991; 78: 154–162.

31 Kieffer N, Phillips DR. Platelet membrane glycoproteins: Functions in cellular interactions. *Annu Rev Biol* 1990; 6: 329–357.

32 Vicente V, Houghten RA, Ruggeri ZM. Identification of a site in the a chain of platelet glycoprotein Ib that participates in von Willebrand factor binding. *J Biol Chem* 1990; 265: 274–280.

33 Fujimura Y, Titani K, Holland LZ et al. von Willebrand factor. A reduced and alkylated 52/48-kDa fragment beginning at amino acid residue 449 contains the domain interacting with platelet glycoprotein Ib. *J Biol Chem* 1986; 261: 381–385.

34 Sosnoski DM, Emanuel BS, Hawkins AL et al. Chromosomal localization of the genes for the vitronectin and the fibronectin receptors α subunits and for platelet glycoproteins IIb and IIIa. *J Clin Invest* 1988; 81: 1993–1998.

35 Fitzgerald LA, Phillips DR. Calcium regulation of the platelet membrane glycoprotein IIb-IIIa complex. *J Biol Chem* 1985; 260: 11366–11376.

36 Calvete JJ, Henschen A, Gonzalez-Rodriguez J. Complete localization of the intrachain disulphide bonds and the N-glycosylation points in the a subunit of human platelet glycoprotein IIb. *Biochem J* 1989; 261: 561–568.

37 Beer J, Coller BS. Evidence that platelet glycoprotein IIIa has a large disulfide bonded loop that is susceptible to proteolytic cleavage. *J Biol Chem* 1989; 264: 17564–17573.

38 Plow EF, Ginsberg MH, Marguerie GA. Expression and function of adhesive proteins on the platelet surface. In Phillips DR, Shuman MA, eds. *Biochemistry of platelets*. New York: Academic Press; 1986: 225–256.

39 Ruoslahti E, Pierschbacher MD. New perspectives in cell adhesion: RGD and integrins. *Science* 1987; 238: 491–497.

40 Doolittle RF, Watt KWK, Cottrell BA, Strong DD, Riley M. The amino acid sequence of the a-chain of human fibrinogen. *Nature* 1979; 280: 464–467.

41 Kloczewiak M, Timmons S, Lukas TJ, Hawiger J. Platelet receptor recognition site on human fibrinogen. Synthesis and structure–function relationship of peptides corresponding to the carboxyterminal segment of the g chain. *Biochemistry* 1984; 23: 1767–1774.

42 Plow EF, Ginsberg MH. Cellular adhesion: GPIIb–IIIa as a prototypic adhesion receptor. *Prog Thromb Haemost* 1989; 9: 117–156.

43 Smyth SS, Joneckis CC, Parise LV. Regulation of vascular integrins. *Blood* 1993; 81: 2827–2843.

44 Smyth SS, Hillery CA, Parise LV. Fibrinogen binding to purified platelet glycoprotein IIb–IIIa (integrin aIIbb3) is modulated by lipids. *J Biol Chem* 1992; 267: 15568–15577.

45 O'Toole TE, Mandelman D, Forsyth J, Shattil SJ, Plow EF, Ginsberg MH. Modulation of the affinity of integrin aIIbb3 (GPIIb–IIIa) by the cytoplasmic domain of aIIb. *Science* 1991; 254: 845–847.

46 Calvete JJ, Mann K, Schäfer W, Fernandez-Lafuente R, Guisan JM. Proteolytic of the RGD-binding and non RGD-binding conformers of platelet integrin GPIIb/IIIa. Clues for identification of regions involved in receptor's activation. *Biochem J* 1994; 298: 1–7.

47 Fox JEB, Lipfert L, Clark EA, Reynolds CC, Austin CD, Brugge JS. On the role of the platelet membrane skeleton in mediating signal transduction. Association of GPIIb-IIIa, pp60c-src, pp62c-yes, and the p21ras GTPase-activating protein with the membrane skeleton. *J Biol Chem* 1993; 268: 25973–25984.

48 Murad F, Forstermann U, Nakane M et al. The nitric oxide–cyclic GMP signal transduction system for intracellular and intercellular communication. *Adv Second Messenger Phosphoprotein Res* 1993; 28: 101–109.

49 Sakon M, Kambayashi JI, Murata KH. The involvement of protein phosphatases in platelet activation. *Platelets* 1994; 5: 130–134.

50 Brass LF, Hoxie JA, Kieber-Emmons T et al. Agonists receptors and G proteins as mediators of platelet activation. *Adv Exp Med Biol* 1993; 344: 17–36.

51 Brass LF, Hoxie JA, Manning DR. Signaling through G proteins and G protein-coupled receptors during platelet activation. *Thromb Haemost* 1993; 70: 217–223.

52 Brass LF. The biochemistry of platelet activation. In Hoffman R, Benz EJ Jr, Shattil SJ, Furie B, Cohen HJ, eds. *Hematology, basic principles and practice*. New York: Churchill Livingstone; 1991: 1176–1197.

53 Fernandez-Ortiz A, Badimon J, Falk E et al. Characterization of the relative thrombogenicity of atherosclerotic plaque components: implications for consequences of plaque rupture. *J Am Coll Cardiol* 1994; 23: 1562–1569.

54 Toschi V, Gallo R, Lettino M et al. Tissue Factor activity modulates the thrombogenicity of human atherosclerotic plaques. *Circulation* 1997: 95; 594–599.

55 Banner DW, D'Arcy A, Chene C, Winkler FK, Guha A, Konigsberg WH, Nemerson Y, Kirchhofer D. The crystal structure of the complex of blood composition factor VIIIa with soluble tissue factor. *Nature* 1996; 380: 41–46.

56 Moreno PR, Bernadi VH, Lopez-Cuellar J et al. Macrophages, smooth muscle cells and tissue factor in unstable angina; implications for cell mediated thrombogenicity in acute coronary syndromes. *Circulation* 1996; 94: 3090–3097.

57 Badimon JJ, Lettino M, Toschi V et al. Local inhibition of tissue factor reduces the thrombogenicity of disrupted human atherosclerotic plaques: effects of TFPI on plaque thrombogenicity under flow conditions. *Circulation* 1999; 14: 1780–1787.

58 Vu TH, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* 1991; 64: 1057–1068.

Cambridge Books Online © Cambridge University Press, 2009

59 Giesen PLA, Rauch U, Borman B et al. Blood-borne tissue factor: another view of thrombosis. *Proc Nat Acad Sci USA* 1999; 96: 2311–2315.
60 Ridker PM, Blynn RJ, Hennekens CH. C-Reactive protein adds to the predictive value of total and HDL cholesterol in determining risk of first myocardial infarction. *Circulation* 1998; 97: 2007–2011.
61 Ridker PM, Glynn RJ, Hennekens CH. Inflammation, Pravastin and the risk of coronary events after myocardial infarction in patients with average cholesterol levels. *Circulation* 1998; 97: 2007–2011.
62 Koenig W, Sund M, Frohlich M et al. C-Reactive protein, a sensitive marker of inflammation, predicts future risk of coronary heart disease in initially healthy middle-aged men: results from the MONICA (monitoring trends and determinants in cardiovascular disease) Augsbrug Cohort study, 1984 to 1992. *Circulation* 1999; 99: 237–242.
63 Biasucci LM, Liuzzo G, Grillo RL et al. Elevated levels of C-reactive protein at discharge in patients with unstable angina predict recurrent instability. *Circulation* 1999; 99: 855–860.
64 Burke AP, Farb A, Malcom GT, Liang YH, Smialek J, Virmani R. Coronary risk factors and plaque morphology in men with coronary disease who died suddenly. *N Engl J Med* 1997; 336: 1276–1281.
65 Farb A, Burke AP, Tang AL et al. Coronary plaque erosion without rupture into a lipid core: a frequent cause of coronary thrombosis in sudden coronary death. *Circulation* 1996; 93: 1354–1363.
66 Dangas G, Badimon JJ, Smith DA et al. Pravastin therapy in hyperlipidemia; effects on thrombus formation and the systemic hemostatic profile. *J Am Coll Cardiol* 1999; 33: 1294–1304.
67 Rauch U, Osende JI, Chesebro JH et al. Statins and cardiovascular diseases: multiple effects of lipid-lowering therapy by statins. *Atherosclerosis* 2000; 153: 181–189.
68 Libby P, Egan D, Skarlatos S. Roles of infectious agents in atherosclerosis and restenosis: An assessment of the evidence and need for future research. *Circulation* 1997; 96: 4095–4103.
69 Maseri A, L'Abbate A, Baroldi G et al. Coronary vasospasm as a possible cause of myocardial infarction: a conclusion derived from the study of preinfarction angina. *N Engl J Med* 1978; 299: 1271–1277.
70 Bogaty P, Hackett D, Davies G, Maseri A. Vasoreactivity of the culprit lesion in unstable angina. *Circulation* 1994; 90: 5–11.
71 Willerson JT, Golino P, Eidt J, Campbell WB, Buja LM. Specific platelet mediators and unstable coronary artery lesions: experimental evidence and potential clinical implications. *Circulation* 1989; 80: 198–205.
72 Theroux P, Fuster V. Acute coronary syndromes. *Circulation* 1998; 97: 1195–1206.

Cambridge Books Online © Cambridge University Press, 2009

48

Platelets and atherosclerosis

Peter Verhamme and Paul Holvoet

Cardiovascular Research Unit of the Center for Experimental Surgery and Anesthesiology, University of Leuven, Belgium

Cardiovascular diseases remain the principal cause of morbidity and mortality in the Westernized world. Atherosclerosis is the common pathophysiological process underlying ischemic heart disease, cerebrovascular accidents and peripheral vascular disease. Understanding of the pathogenesis of atherosclerosis has tremendously increased in the last decade. For years, atherosclerosis had been considered as a degenerative process of the arteries. Nowadays, atherosclerosis is seen as an inflammatory disease of the artery resulting from chronic endothelial injury. Platelets have proinflammatory and growth-regulatory properties that may contribute to the progression and destabilization of atherosclerotic plaques. Platelets activate endothelium, promote lipoprotein and lipid peroxidation, mediate leukocyte–endothelial interaction and stimulate smooth muscle cell proliferation. An acute clinical syndrome usually involves thrombosis triggered by the rupture of a vulnerable atherosclerotic plaque. Incorporation of mural platelet-rich thrombi at the site of an unstable plaque contributes to the progression of atherosclerotic lesions.

Pathogenesis of atherosclerosis

Response to injury

Healthy endothelium maintains vascular integrity. It provides a non-thrombogenic inner layer of the vessel wall, and tends to dilate the vessel by releasing endothelium-derived relaxing factor (nitric oxide, NO), endothelium-derived hyperpolarizing factor (EDHF) and prostacylin (PGI2). NO and PGI2 both inhibit the adhesion and aggregation of platelets. Furthermore, healthy endothelium has fibrinolytic properties and impedes leukocyte adhesion[1].

Chronic endothelial injury is a key feature in the pathogenesis of atherosclerosis. Cardiovascular risk factors such as dyslipidemia, hypertension, smoking, diabetes, hyperhomocysteinemia and systemic infection or inflammation result in increased oxidative stress of the vascular wall, endothelial dysfunction and platelet hyperactivity (Fig. 48.1)[2]. High levels of high-density lipoproteins (HDL), moderate alcohol consumption, intake of (ω-3 fatty acids and antioxidants are associated with lower cardiovascular risk and platelet hypoactivity.

Local variations in shear stress and flow help explain the focal distribution of atherosclerosis. It occurs preferentially at lesion prone sites of large and medium-sized elastic arteries (aorta, carotid and iliac bifurcation) and some of the large and medium-sized muscular arteries (coronary arteries, circle of Willis). Lesion prone sites are characterized by decreased shear stress because of oscillatory blood flow at branching vessels or curvatures. Normal arterial shear stress induces an atheroprotective gene expression profile with high expression of NO-synthase and cyclooxygenase. Endothelium exposed to decreased shear stress expresses a more atherogenic phenotype with increased expression of intercellular adhesion molecule-1 (ICAM-1), monocyte chemotactic protein (MCP-1), vascular cell adhesion molecule-1 (VCAM-1) and platelet-derived growth factor (PDGF)[3].

One of the first hallmarks of endothelial activation is increased permeability, leading to enhanced retention of low-density lipoproteins (LDL) (Fig. 48.2). In the vessel wall LDL becomes deprived of antioxidants and undergoes minimal oxidative changes. Oxidized LDL (Ox-LDL) interferes with the NO signalling pathway through many possible mechanisms, including decreased transcription of NO-synthase and inactivation of NO[4,5]. Decreased PGI2 and NO result in decreased vasodilating and antiadhesive properties of the endothelium. Endothelial injury results in decreased anticoagulant properties by increasing Von

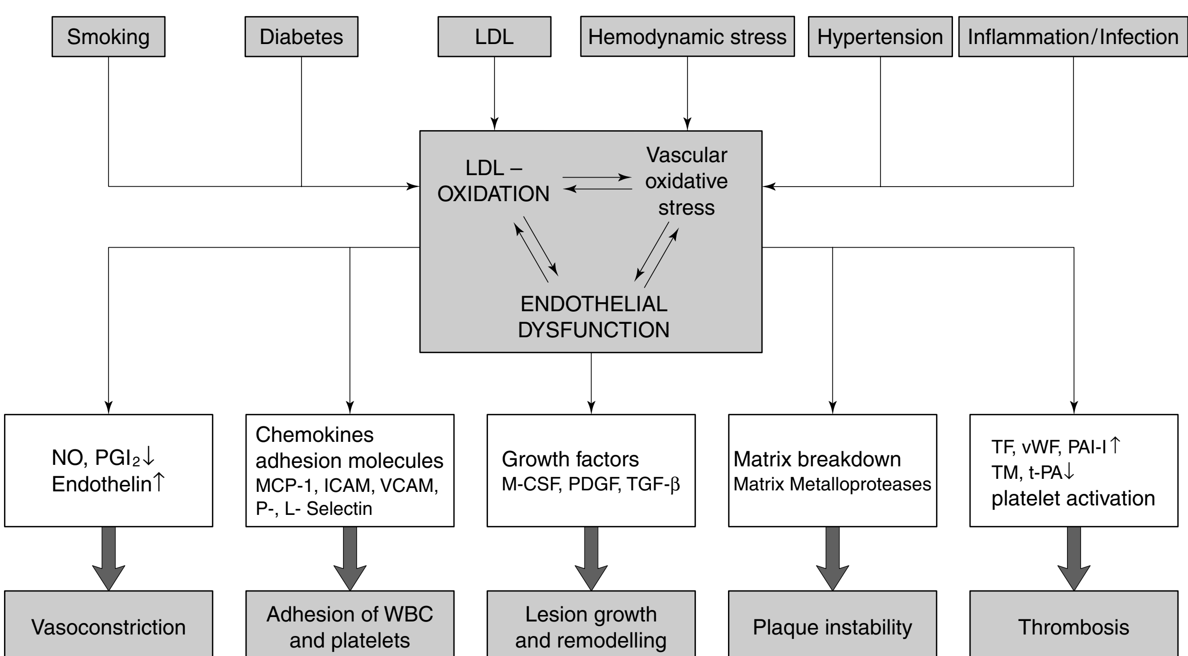


Fig. 48.1. Pathogenesis of atherothrombosis

Willebrand factor (vWF) expression and decreasing the expression of thrombomodulin. Reduced tissue-type plasminogen activator (t-PA) and increased plasminogen activator inhibitor (PAI-1) expression impair fibrinolysis. Minimally oxidized LDL contains oxidized lipids that activate the nuclear factor kappa B family (NFκB) of transcription factors. This redox-regulated transcription factor plays an important role in the chronic inflammation of the vessel wall[6]. It regulates a variety of genes encoding for cytokines (MCP-1, tumour necrosis factor-α) and adhesion molecules (ICAM-1, VCAM-1) important in macrophage adhesion and infiltration[7]. Other transcriptional factors influenced by the cellular redox state and important in the inflammatory reaction of the vessel wall are peroxisome proliferator-activated receptors (PPARs) and activator protein-1 (AP-1)[8].

Inflammatory reaction and recruitment of leukocytes

Endothelial cell activation enhances the adhesion of leukocytes to the vessel wall. MCP-1 and other chemokines such as monocyte chemotactic protein-4 (MCP-4), osteopontin and interleukin-8 are chemotactic molecules for monocytes. Studies with MCP-1 and CCR2-receptor knockout mice revealed that MCP-1 and its main chemokine receptor CCR2 play an important role in the early recruitment of monocytes into the vessel wall[9,10]. Stromal cell-derived factor-1 (SDF-1) is highly expressed in human atherosclerotic plaques. It is not only a chemotactic factor for lymphocytes and macrophages but also induces platelet activation[11].

Homing of leukocytes to the vascular endothelium involves rolling, activation and firm adhesion. Rolling of leukocytes is mediated by E- and P-selectins on endothelial cells, and L-selectins on leukocytes[12,13]. Firm adhesion involves (β_2-integrin interaction and requires activation of the leukocytes by P-selectin or platelet activating factor (PAF)[14]. Platelet endothelial cell adhesion molecule-1 (PECAM) is important in the migration of monocytes into the vessel wall[15]. Monocyte–endothelial cell interaction induces the synthesis of colony stimulating factors, which stimulate further chemotaxis, proliferation, and monocyte differentiation[16].

Resting platelets do not interact with healthy endothelium, but do adhere to activated endothelium at sites of disturbed shear stress and to apoptotic endothelial cells.

Cambridge Books Online © Cambridge University Press, 2009

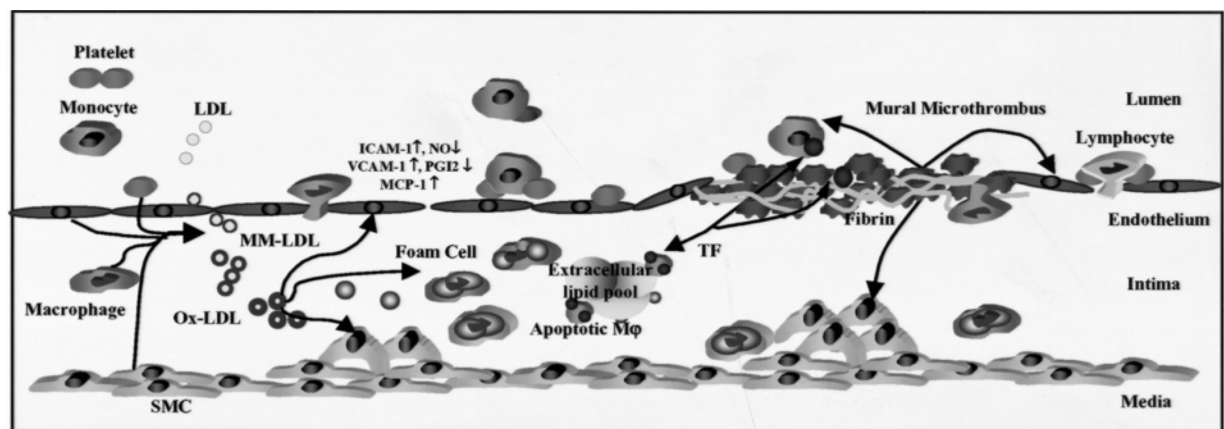
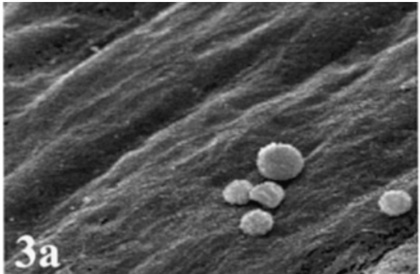


Fig. 48.2. Endothelial cells, platelets, smooth muscle cells and macrophages mediate oxidative modifications of LDL. MM-LDL activates endothelial cells and promotes smooth muscle cell migration and proliferation. Uptake of oxidized lipoproteins by macrophages and smooth muscle cells generates foam cells. Activated platelets adhere to, and activate, endothelium and facilitate monocyte adhesion. Endocytosis of platelet-derived vesicles contributes to foam cell formation. Endothelial erosion initiates platelet adhesion and aggregation and fibrin deposition on the subendothelial matrix. Tissue factor is closely associated with macrophages in the lipid core. Propagation of a thrombus may also depend on blood-borne tissue factor. Platelets release a number of growth factors propagating smooth muscle cell proliferation. Re-endothelialization and cap formation by smooth muscle cells incorporates the mural thrombus into the expanded lesion. (ICAM-1: intercellular adhesion molecule-1, Mψ: macrophage, MCP-1: monocyte chemotactic protein-1, MM-LDL: Minimally modified LDL, NO: nitric oxide, ox-LDL: oxidized LDL, PGI_2: prostacyclin, SMC: smooth muscle cell, TF: tissue factor, VCAM-1: vascular cell adhesion molecule-1.)



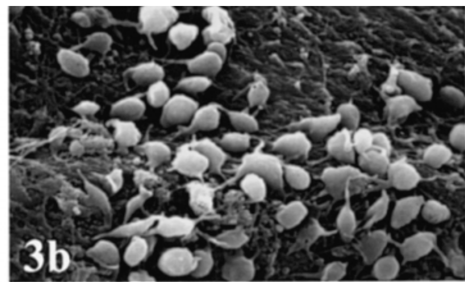


Fig. 48.3. (*a*) Scanning electron microscopy showing platelet adhesion to a dysfunctional endothelium from a hypercholesterolemic rabbit. (*b*) Scanning electron microscopy demonstrating platelet interaction with a damaged arterial wall. Platelets adhere, get sticky and release their granules. (Illustrations kindly provided by Marc Hoylaerts and Carine Michiels.)

Activated platelets adhere to both healthy and activated endothelium (Fig. 48.3)[17]. Platelet rolling on activated endothelium is mediated by endothelial P-selectin. Firm interaction of activated platelets with endothelium involves GPIIb–IIIa-dependent bridging and ICAM-1, GPIbα and $\alpha_V\beta_3$ integrin interaction[18,19]. Activated platelets release proinflammatory cytokines, chemokines and lipid metabolites and are capable of inducing lipid peroxidation[20,21]. They express CD40 ligand, involved in inflammatory signalling, and P-selectin. CD40–CD40L signalling activates atheroma-associated cells and appears to play a key role in the progression of atherosclerotic lesions[22]. Inhibition of CD40 signalling significantly reduces atherosclerosis in mice[23]. Via CD40 ligand, activated platelets trigger an inflammatory response in endothelial cells[24]. They induce expression of adhesion molecules (ICAM-1, VCAM-1), of chemokines (IL-6, MCP-1 and IL-8) and of tissue factor (TF)[25,26].

Activated platelets also interact with leukocytes and trigger leukocyte activation, expression of NFκB regulated gene products, expression of adhesion receptors, e.g. Mac-1 and shedding of L-selectin[27,28]. P-selectin is the major surface receptor for leukocytes on activated platelets and interacts with P-selectin glycoprotein ligand that is abun-

Cambridge Books Online © Cambridge University Press, 2009

Table 48.1. Platelet involvement in atherothrombosis

Early lesion	Activated platelets adhere to endothelium and may: • induce lipid and lipoprotein peroxidation generating a broad range of biological active lipids[21]. • trigger an inflammatory response in endothelium by CD40–CD40 ligand interaction and NFκB activation[24]. • increase expression of adhesion molecules and secretion of chemokines[26]. • facilitate adhesion of monocytes by P-selectin mediated interaction with adherent platelets[30]. Circulating monocyte–platelet conjugates may facilitate monocyte homing to the arterial wall[39]. Interaction of platelets and macrophages enhances foam cell formation.
Lesion progression	Plaque rupture or endothelial erosion triggers platelet adhesion, activation and aggregation and the formation of mural thrombi that may: • incorporate in the plaque causing rapid expansion of the lesion[63]. • release growth factors from α-granules inducing SMC proliferation, migration and differentiation[41]. • enhance adhesion and infiltration of leukocytes[74,167].
Thrombosis and clinical events	Occluding platelet-rich thrombi are formed upon rupture of a vulnerable plaque[52]. Embolization of platelet aggregates is a possible cause of ventricular fibrillation and sudden death and limb gangrene[60]. Arterial spasm can be triggered by vasoconstrictors released from activated platelets[168,169]. Activated platelets contribute to a procoagulant state[25,33].

dant on circulating monocytes and lymphocytes[29,30]. Increased soluble P-selectin among patients with atherosclerosis may reflect both platelet activation and endothelial dysfunction[31]. Furthermore, activated platelets induce TF expression in human circulating monocytes and thus contribute to a systemic procoagulant state[32,33]. Triggering of TF on circulating monocytes by activated platelets does not depend on *de novo* protein synthesis but on translocation from intracellular stores[34,35].

Adherent platelets may trap circulating lymphocytes or monocytes and propagate the adherence of leukocytes to the vessel wall even at shear stress that does not allow direct leukocyte adhesion[36–38]. Furthermore, in vitro studies suggest that rosette formation of activated platelets with monocytes or lymphocytes may facilitate adhesion of these conjugates to the vessel wall[39,40]. (see also Table 48.1 and Fig. 48.2).

Fatty streak

Recruitment of monocytes, lymphocytes and platelets to the vessel wall initiates an inflammatory response. Infiltration and proliferation of monocytes and T-lymphocytes in the subendothelial space is induced by a variety of growth-regulatory molecules released by endothelial cells, platelets, smooth muscle cells, and leukocytes[41]. Macrophages, endothelial cells, platelets and smooth muscle cells may further oxidize LDL. Oxidation of LDL may be catalysed by myeloperoxidase or lipoxygenase, or initiated by metal ions. Further oxidation of LDL results in the generation of lipid mediators such as lysophosphatidylcholine (LPC), oxysterols, and PAF-like molecules that have chemotactic properties towards macrophages and further activate endothelial cells[42]. Oxidized lipoproteins initiate an immune response. Ox-LDL activates T-lymphocytes in humans[43] and titres of plasma autoantibodies to ox-LDL are higher in patients with carotid atherosclerosis[44].

Oxidative alterations of the protein moiety of LDL result in a shift from LDL receptor binding to scavenger receptor binding. Contrary to the LDL receptor, scavenger receptors are not downregulated by intracellular cholesterol pools, resulting in an uncontrolled uptake of ox-LDL and foam cell formation[45]. Histological studies have shown platelets and foam cells in close proximity[46]. Interaction between platelets and macrophages enhances foam cell formation[47]. Activated platelets or microvesicles shed from activated platelets stimulate cholesterol esterification and cholesterol ester accumulation in macrophages. Part of the lipid content in foam cells may be derived from endocytosis of platelets or platelet-derived vesicles[48]. Accumulation of foam cells is the hallmark of the earliest recognizable lesion, the fatty streak (Fig. 48.4(*a*), see colour plate). It is very common in young children and adolescents and is still a reversible stage of atherosclerosis[49]. It consists mainly

Cambridge Books Online © Cambridge University Press, 2009

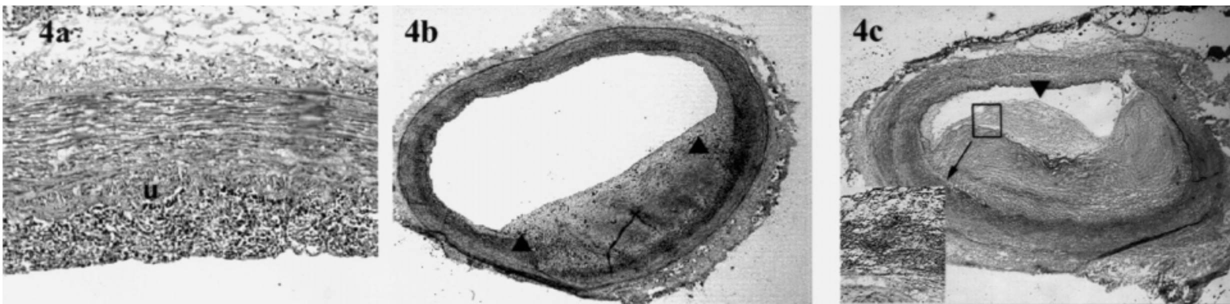


Fig. 48.4. (*a*) Oil Red O staining of a fatty streak (Stary type II lesion). The lesion is rich in foam cells. Smooth muscle cells (▶) migrate towards the intima.(*b*) Oil Red O staining of a fibroatheroma (Stary type IV–V). The eccentric lesion consists of a large lipid core with a rather thin fibrous cap. Plaque rupture occurs most frequently at the shoulder regions of the plaque (▲). (*c*) Oil Red O staining of a complicated lesion (Stary type VI). The layered aspect and the organized thrombus (▼) are hallmarks of healed disruption. Immunostaining (➔) for fibrin shows fibrin at the basis of the most recently formed cap.

of inflammatory cells, predominantly macrophages, macrophage-derived foam cells and T-cells. Uptake of ox-LDL by macrophages is initially considered to be protective, minimizing the inflammatory and cytotoxic effects of oxidized lipids on endothelial and smooth muscle cells and promoting lipid efflux out of the vessel wall.

Fibroatheroma

Ongoing endothelial injury and unregulated accumulation of oxidized lipids lead to necrosis and apoptosis of intimal macrophages and release of oxidized lipids that mitigate further inflammatory and cytotoxic effects. Confluence of extracellular lipid pools results in the formation of a lipid core. As a result of ongoing inflammation, and enhanced by oxidized lipids, macrophages, smooth muscle cells and platelets, together with the endothelium release a variety of growth-regulatory molecules. Smooth muscle cells migrate and proliferate to form a fibrous cap covering the lipid core and synthesize extracellular matrix (Fig. 48.4(*b*), see colour plate). Smooth muscle cells stimulated to express scavenger receptors become foam cells by uptake of ox-LDL. As the lesion grows, plaque vascularization is an additional source of inflammatory cells and can give rise to in-plaque hemorrhage. Calcification is a common feature of advanced atherosclerotic plaques. Calcifying vascular cells are stimulated by transforming growth factor-β (TGF-β) and oxidized lipids[50]. Plaque progression does not necessarily decrease lumen size through adaptive remodelling of the vessel. Studies with intravascular ultrasound have revealed that large atherosclerotic lesions can be present in the coronary arteries before luminal narrowing is detectable with coronary angiography. Although mechanisms for adaptive remodelling remain largely unravelled, it is generally accepted that it is shear stress and NO dependent[51].

The unstable lesion

Lesions that are prone to rupture are best characterized in the coronary arteries. A lipid-rich core underlying a thin fibrous cap is the histological hallmark of an unstable plaque. Shoulder regions are heavily infiltrated by inflammatory cells, mainly T-cells and macrophages, and are most prone to plaque rupture (Fig. 48.4(*b*), see colour plate)[52]. Matrix metalloproteases secreted and activated by macrophages and T-lymphocytes degrade the extracellular matrix[53]. Imbalance between matrix synthesis and breakdown causes weakening of the plaque. Plaque rupture exposes flowing blood to the thrombogenic core. Thrombogenicity of the lipid core is attributed to TF that is closely associated with apoptotic cells in the lipid core[54]. Oxidized LDL stimulates the expression of TF in both macrophages and endothelial cells[55,56].

Acute vascular events

Atherosclerosis is a systemic disease with a broad range of clinical manifestations. An acute coronary syndrome is triggered by an occluding platelet-rich thrombus that is formed upon a vulnerable plaque. Most frequently, an unstable plaque shows rupture into the lipid core, although coronary plaque erosion is also a common cause of coronary thrombosis and sudden death[57]. Remarkably, in about 75% of patients with acute coronary syndromes, the culprit lesion shows only mild to moderate stenosis on

Cambridge Books Online © Cambridge University Press, 2009

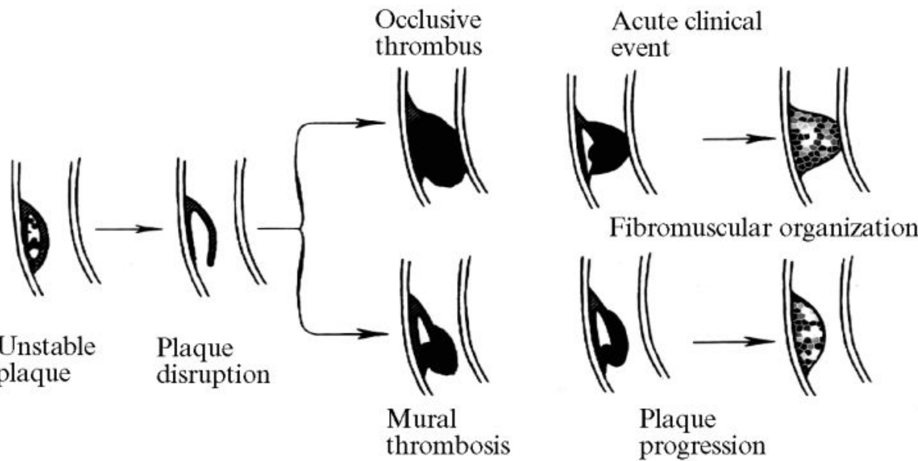


Fig. 48.5. Typical evolution of the unstable lesion. Plaque rupture triggers formation of a platelet-rich thrombus. Mural thrombi contribute to lesion progression and are far more common than occlusive thrombi that may lead to acute clinical events. (Adapted from V. Fuster[61].)

angiography. Total occlusion of a coronary artery may result in myocardial infarction. Fluctuating and recurrent thrombus formation in the coronary arteries with intermittent thrombus dissemination is the underlying mechanism for unstable angina. Markers for platelet activation are increased among patients with unstable angina, confirming ongoing thrombosis[58,59]. Adherent platelets may release vasoconstrictors mitigating coronary spasm. Distal embolization of the platelet aggregates in the myocardium is a potential cause of ventricular fibrillation and sudden death[60]. Ischemic cerebrovascular accidents are caused by occlusion or thromboembolism of vulnerable plaques in the aorta, carotid arteries or cerebral arteries. Claudication and gangrene of the lower limbs are the clinical manifestations of peripheral artery disease or thromboembolic phenomena from the aorta.

Mural thrombi

Prevalence of mural thrombi

The role of plaque vulnerability and thrombus formation in acute clinical syndromes is well recognized. Plaque instability and mural thrombosis also play a crucial role in the progression of atherosclerotic lesions (Fig. 48.5)[61]. Platelet adhesion and thrombosis are most frequently initiated by plaque disruption, but platelet-rich thrombi may also be formed at sites of activated, apoptotic or eroded endothelium. Histopathological studies have demonstrated that plaque instability and mural thrombosis are very common in coronary atherosclerosis. Postmortem studies revealed plaque disruption in 17% of patients who suddenly died because of a non-cardiac event[62]. Analysis of coronary arteries demonstrated healed disruption in 16% of atherosclerotic lesions with up to 20% stenosis, 19% of lesions with 24–50% stenosis, and 73% of lesions with more than 50%[63] stenosis. These studies stress the importance of plaque rupture and incorporation of mural thrombi with subsequent fibrotic organization in the progression of atherosclerotic plaques and help explain the episodes of rapid plaque growth and non-linear progression of atherosclerosis frequently observed among patients.

Mural versus occluding thrombi

Mural thrombosis is far more frequent than occlusive thrombosis. The extent of thrombus formation may depend on local variations in thrombogenicity of the ruptured lesion, local production of platelet activators, degree of endothelial dysfunction with impaired fibrinolysis and reduced release of platelet antagonists (NO, PGI2) and local shear stress[64]. It may also depend on systemic variations in thrombotic tendency and fibrinolytic capacity that are of environmental origin or genetically determined. Many of the proteins involved in coagulation and fibrinolysis might contribute to a thrombotic tendency. High circulating PAI-1 is an independent risk factor for recurrent myocardial infarction[65]. High factor VII, fibrinogen and vWF increase the risk of myocardial infarction[66–68]. Platelet hyperactivity also contributes to a thrombotic tendency. Well-known risk factors for atherothrombosis are associated with platelet hyperresponsiveness and will be discussed later in this chapter.

Cambridge Books Online © Cambridge University Press, 2009

Platelets of patients suffering from an acute coronary syndrome produce less NO[69]. Platelets contain both constitutive and inducible NO synthase and platelet-derived NO prevents further platelet recruitment[70,71]. Impaired platelet-derived NO production may contribute to the development of an occluding thrombus. Concurrent increase in platelet aggregability and risk of myocardial infarction early in the morning further suggests a role of platelet reactivity in the onset of myocardial infarction[72].

Incorporation of mural thrombi and plaque growth

Plaque disruption or superficial erosion initiates fibrin deposition and platelet adhesion to the sub-endothelial matrix with subsequent activation and aggregation (Fig. 48.3(*b*)). Platelet receptors involved in aggregate formation and matrix adhesion have been studied extensively[73]. vWF plays a critical role in mediating platelet adhesion to the arterial subendothelium. Initial platelet–vWF interactions are mediated primarily by GPIb and are consolidated by GPIIb/IIIa. Activated platelets and fibrin deposits may allow circulating leukocytes to adhere even at high shear rates, propagating the influx of leukocytes into the evolving thrombus and the underlying lesion[74]. P-selectin expressed on surface-bound platelets mediates leukocyte rolling. Firm adhesion requires integrin–ligand interactions. Mac-1 is the main leukocyte (β_2-integrin that binds to the platelet surface. Intercellular adhesion molecule-2 on platelets and fibrin are the main ligands for Mac-1[40,75]. Platelet–leukocyte cross-talk is important in the propagation of the thrombus. Thrombus formation may not only be dependent on vascular TF but also on the deposition of blood-borne TF shed from monocytes in microvesicles after interaction with platelets[35].

Platelet adhesion and mural thrombosis stimulate smooth muscle cell migration and proliferation. Platelets have growth regulatory properties for smooth muscle cells by the release from α-granules of platelet derived growth factor (PDFG), epidermal growth factor (EGF), insulin-like growth factor (IGF) and transforming growth factor-β (TGF-β). Serotonin released by activated platelets stimulates the expression of thrombin receptors on smooth muscle cells, thereby potentiating the mitogenic and vascoconstrictor actions of thrombin[76]. Thromboxane not only causes platelet aggregation and vasoconstriction but also induces chemotaxis of smooth muscle cells. Pigs with von Willebrand disease are resistant to atherosclerosis most likely due to reduced platelet–vessel wall interaction[77].

After re-endothelialization and cap formation by smooth muscle cells, the clot gets incorporated into the expanded lesion and undergoes further fibrotic organization (see also Fig. 48.4(*c*) and Table 48.1).

Platelets and restenosis

Angioplasty of a narrowed or occluded coronary or peripheral artery is common strategy to treat patients with coronary and peripheral artery disease. Antiplatelet and antithrombotic drugs have decreased the rate of acute occlusion of the vessel after the intervention. However, restenosis after 3 to 6 months remains a clinical problem complicating about 30% of all coronary interventions.

Rupturing the atherosclerotic vessel wall by balloon dilation promotes the adhesion and aggregation of platelets and thrombus formation (Fig. 48.3(*b*)). Activated platelets release their granules with growth factors and cytokines that promote smooth muscle cell migration and proliferation. The thrombus provides a matrix for smooth muscle cell growth[78,79]. In animal models of balloon injury, neointima formation can be retarded by anti-platelet therapy. Thrombocytopenic rabbits have reduced neointima formation after balloon injury[80]. Cyclic RGD-containing peptides and GPIIb/IIIa antagonists reduce neointima formation in animal models[81].

Antiplatelet strategies had however little effect on restenosis in humans. Constrictive remodelling of the injured vessel, rather than neointimal growth may be the major determinant of human restenosis. Stents inhibit constrictive remodelling of the vessel wall[82]. Antiplatelet therapy effectively prevents acute and subacute *in stent* thrombosis[83].

Platelet count, platelet activity and atherothrombosis

There are few arguments to consider absolute platelet count as a risk factor for atherothrombosis. In one prospective study of 487 apparently healthy men, subjects in the highest quartile of platelet count had a 2.5-fold increased risk of cardiovascular mortality within 10 years[84]. Platelet function may be more relevant than absolute number. In the *Caerphilly Collaborative Heart Disease* study, platelet aggregation was measured in 2000 men without recent history of aspirin intake. Coronary heart disease patients had significantly greater ADP-induced platelet aggregation compared with controls[85,86]. Increased platelet sensitivity to ADP in men without clinically overt ischemic heart disease was associated with increased risk of cardiovascular mortality within 13 years of follow-up[84].

Cambridge Books Online © Cambridge University Press, 2009

In the *Caerphilly Cohort* study, thrombin and ADP-induced platelet aggregation were not predictive of ischemic heart disease[87]. Among patients with myocardial infarction, spontaneous platelet aggregation was a useful predictor for survival and future coronary events within 5 years[88]. Platelet size is also shown to be a predictor of recurrent myocardial infarction and death. Men with a fatal or non-fatal myocardial infarction within two years after a first infarction had a greater mean platelet volume 6 months after the first event than men without recurrent coronary syndromes. An increased mean platelet volume is associated with increased platelet activity and may reflect an enhanced inflammatory state[89].

The most convincing evidence that platelets are involved in atherothrombosis arises from trials with antiplatelet drugs[90]. Small amounts of aspirin, sufficient to irreversibly inhibit cyclooxygenase in platelets, reduce the risk of non-fatal MI, non-fatal stroke and vascular death among patients with previous cardiovascular disease. The Physicians Health Study demonstrated a conclusive and significant reduction of a first myocardial infarction in physicians without a history of cardiovascular disease randomized to aspirin after 5 years of follow-up[91]. The strong negative relation between alcohol intake and consumption of ω-3 fatty acids and platelet aggregation may partially explain the lower incidence of ischemic heart disease in patients consuming a moderate amount of red wine or fish oil[92–95].

Platelet activity and risk factors of atherothrombosis

There is a need for a measure of platelet activity that is predictive of ischemic vascular disease and that more effectively guides antiplatelet therapy. Many assays have been proposed to determine the degree of platelet activation. Besides spontaneous platelet aggregation or aggregability using different agonists, platelet factor 4 (PF4), soluble P-selectin, β-thromboglobulin, platelet size and heterogeneity, and metabolites of thromboxane/prostacyclin synthesis are used to assess platelet activity[96]. Flow cytometry of platelets in whole blood enables investigation of functional aspects of circulating platelets by assessment of their membrane glycoproteins[97]. Other markers may indirectly reflect platelet activation. Isoprostanes are markers for lipid peroxidation in vivo. Oxidative stress results in the generation of F2-isoprostanes that are non-cyclooxygenase free radical catalysed oxidation products of arachidonic acid. They form *in situ* on esterified phospholipids of cell membranes and LDL particles and are subsequently released through phospholipases[98,99]. F2-isoprostanes are strong aggregatory and vasoconstricting agents. Their levels increase with age, in smokers, diabetics and hypercholesterolemic patients, and may contribute to persistent platelet activation in those patients[100–103].

Table 48.2. Atherogenic and atheroprotective factors associated with platelet hyper-responsiveness and platelet hyporesponsiveness

Platelet hyperactivity	Platelet hypoactivity
LDL, ox-LDL[127,133,170]	HDL[126]
Smoking[109]	Alcohol intake[172]
Diabetes[112,115]	Intake of ϖ-3 fatty acids[94]
Hypertension[104,105]	Antioxidant therapy[150]
Hyperhomocysteinemia[107]	Antiplatelet agents (e.g. aspirin)[90]
Ageing[171]	

Most of the conventional cardiovascular risk factors are associated with increased platelet responsiveness (Table 48.2). Systemic activation of circulating platelets and endothelial dysfunction may be closely interdependent and favourize thrombosis after plaque rupture.

Platelets are in an activated state in patients with hypertension, the degree of activation correlating with diastolic blood pressure[104,105]. Accelerated aggregation of platelets is demonstrated in patients with hyperhomocysteinemia[106,107]. Homocysteine enhances platelet adhesion to endothelial cells under flow conditions[108]. Smoking is associated with platelet hyperactivity[109], which is corrected after smoking cessation, but also with impaired fibrinolysis[110] and increased TF expression[111].

Coronary and peripheral artery disease is the major cause of morbidity and mortality in diabetics. Diabetes is associated with platelet hyperactivity and increased surface expression of P-selectin[112–115]. The occurrence of renal and vascular complications of diabetes mellitus correlates with the presence of advanced glycation end products (AGEs)[116]. AGEs not only enhance the aggregation of human platelets ex vivo and inhibit PGI2 synthesis[117,118], they also induce TF expression in monocytes[119] and cellular lipid peroxidation[120]. Increased plasma levels of isoprostanes[121], thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides further suggest increased oxidative stress in diabetic patients. Increased isoprostanes correlate with increased platelet activation, while improved metabolic control in diabetic patients reduces both lipid peroxidation and platelet activation[102]. Long-term administration of antiplatelet drugs to patients with type 2 diabetes attenuates the progression of carotid atherosclerosis[122].

Cambridge Books Online © Cambridge University Press, 2009

LDL is a major risk factor for cardiovascular disease. The interaction between lipoproteins and platelets is complex and incompletely understood[123]. Hypercholesterolemic patients display persistent platelet activation in vivo, increased sensitivity of platelets to aggregating agents in vitro, and increased levels of soluble P-selectin[124,125]. The platelet aggregating properties of native LDL itself are controversial. Some studies found no aggregating effect of native LDL[126,127], while others demonstrated platelet-stimulating effects of LDL[128–130]. Ex vivo LDL oxidation during LDL isolation may have troubled these findings since mild oxidation of LDL produces a wide range of biologically active lipids such as lysophosphatidylcholine, oxysterols, F_2-isoprostanes, PAF-like lipids and lysophosphatidic acid (LPA). Whereas fully oxidized LDL is abundant in atherosclerotic plaques and has important cytotoxic effects, circulating levels of ox-LDL and oxidized lipids have been demonstrated in both patients with coronary artery disease and in animal models of atherosclerosis and may directly influence platelet behavior[131,132]. Mildly oxidized LDL is more capable of activating platelets than native LDL[127,133–135]. Mildly oxidized LDL induced platelet aggregation involves phospholipase A_2 (PLA_2) and cyclooxygenase (COX) and is inhibited by aspirin[133]. Mildly oxidized LDL interacts with the lysophosphatidic acid (LPA) receptor and induces platelet shape change via the GTPase Rho and its effector molecule p160 Rho-kinase leading to phosphorylation of the myosin light chain[136,137]. Furthermore, ox-LDL decreases L-arginine uptake and NO synthase activity in platelets[138]. Endothelial dysfunction with reduced basal release of NO and PGI2 may also contribute to increased platelet activation in hypercholesterolemic patients. Platelet activation can be reversed by diet, lipid lowering[139] and administration of NO donors or L-arginine[140,141]. L-Arginine supplementation decreases platelet reactivity by directly providing the substrate for NO-synthase in platelets and by restoring endothelium derived NO activity.

Stabilization of atherosclerotic lesions

Since atherosclerosis is the result of various forms of chronic injury of the endothelium, lesions can at least partially be reversed or stabilized if the injurious agents are removed. Smoking cessation, improved metabolic control in diabetics and lipid lowering not only reduce arterial inflammation and improve endothelial function, but also influence platelet behaviour.

Lipid lowering

Lipid lowering reduces cardiovascular mortality and morbidity[142]. Lipid efflux from plaques decreases the arterial inflammation and retards matrix breakdown, improving the mechanical strength of the plaque[143]. It reduces TF expression in rabbit atherosclerotic plaque[144]. It improves endothelial antiaggregatory and fibrinolytic properties and decreases platelet reactivity. The beneficial effect of statins may partially be due to reduction of thrombotic risk and inhibition of progression of plaques by platelet-dependent processes. Both in vivo and in vitro studies have reported that most of the statins are able to reduce platelet aggregation[145–147]. Atorvastatin significantly inhibits platelet deposition on a mildly damaged vessel wall at high shear rates in hypercholesterolemic pigs[148].

Antioxidant therapy

Antioxidant therapy aiming at reducing oxidative stress and LDL oxidation in the vessel wall seems promising. In numerous animal models of atherosclerosis, therapy with α-tocopherol, β-carotene or other antioxidants retarded atherosclerosis. In humans, dietary intake of antioxidants is inversely correlated with cardiovascular risk[149]. Antioxidant supplementation reduces platelet activation[150], increases the resistance of LDL towards oxidation and improves endothelial function. However, antioxidant supplementation failed to demonstrate clinical benefit in large randomized trials[151–153]. Antioxidant therapy is still controversial[154] and warrants further investigation[153].

HDL

Epidemiological evidence has established an inverse relation between plasma HDL cholesterol levels and risk of coronary heart disease. The reverse cholesterol transport is the major proposed mechanism for the protective effect of HDL in atherothrombosis. HDL may deplete atherosclerotic plaques of cholesterol by promoting efflux of lipids. Other HDL properties may also significantly contribute to the atheroprotective effects of HDL. HDL acts as an antioxidant and inhibits oxidative modification of LDL. Two HDL-associated enzymes, paraoxonase (PON) and platelet activating factor–acetyl hydrolase (PAF–AH) prevent the oxidation of LDL. PON-deficient mice are more susceptible to atherosclerosis[155]. Overexpression of PAF–AH reduces macrophage homing in apolipoprotein E deficient mice[156]. HDL has anti-inflammatory capacities and in vitro studies show that apolipoprotein A-I, the major HDL-associated apolipoprotein, inhibits the expression of adhesion mole-

Cambridge Books Online © Cambridge University Press, 2009

cules on endothelial cells. Furthermore, HDL may play an active role in the modulation of platelet function[157]. HDL inhibits platelet aggregation in vitro[158] and apolipoprotein A-I is a prostacyclin stabilizing factor[159]. Apolipoprotein A-I Milano delayed platelet aggregation and thrombus formation after vascular injury in Sprague–Dawley rats[160].

Future developments

Genetic factors determine the susceptibility for atherothrombosis. The risk of myocardial infarction in subjects with a first-degree relative with CAD is seven times the risk of subjects without family history of cardiovascular disease[161]. Unravelling the common genetic polymorphisms involved in atherothrombosis will further improve the assessment of cardiovascular risk. Several platelet membrane proteins have two or more allelic forms. In a case-control study, the PlA2 allele of the PlA1/A2 polymorphism of the platelet glycoprotein IIIa (GPIIIa) gene was associated with the prevalence of acute vascular events before the age of 60[162]. However, these findings could not be confirmed in a larger cohort study[163].

Targeting of genes via homologous recombination in embryonic stem cells or stem cell injection has allowed the generation of knockout and transgenic mice (for review see ref.[164]). Although these genetically modified mice have already revealed important pathophysiological mechanisms of atherothrombosis, conditional or tissue-specific gene expression will further improve insight in mechanisms of atherothrombosis.

As yet patients with vulnerable plaques cannot be identified with great sensitivity. Culprit lesions often cause only mild to moderate stenosis and are not yet detectable with any current diagnostic tool. Core size and composition and cap thickness rather than the absolute plaque size determines vulnerability[165]. Better identification of patients with lesions prone to rupture will allow better prevention. Nuclear magnetic resonance seems promising for assessment of lesion distribution and composition[166].

References

1 Harrison DG. Endothelial dysfunction in atherosclerosis. *Basic Res Cardiol* 1994; 89 Suppl 1: 87–102.

2 Glasser SP, Selwyn AP, Ganz P. Atherosclerosis: risk factors and the vascular endothelium. *Am Heart J* 1996; 131: 379–384.

3 Malek AM, Alper SL, Izumo S. Hemodynamic shear stress and its role in atherosclerosis. *JAMA* 1999; 282: 2035–2042.

4 Chin JH, Azhar S, Hoffman BB. Inactivation of endothelial derived relaxing factor by oxidized lipoproteins. *J Clin Invest* 1992; 89: 10–18.

5 Liao JK, Shin WS, Lee WY, Clark SL. Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. *J Biol Chem* 1995; 270: 319–324.

6 Thurberg BL, Collins T. The nuclear factor-kappa B/inhibitor of kappa B autoregulatory system and atherosclerosis. *Curr Opin Lipidol* 1998; 9: 387–396.

7 Berliner JA, Territo MC, Sevanian A et al. Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *J Clin Invest* 1990; 85: 1260–1266.

8 Kunsch C, Medford RM. Oxidative stress as a regulator of gene expression in the vasculature. *Circ Res* 1999; 85: 753–766.

9 Aiello RJ, Bourassa PA, Lindsey S et al. Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 1999; 19: 1518–1525.

10 Kuziel WA, Morgan SJ, Dawson TC et al. Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. *Proc Natl Acad Sci USA* 1997; 94: 12053–12058.

11 Abi-Younes S, Sauty A, Mach F, Sukhova GK, Libby P, Luster AD. The stromal cell-derived factor-1 chemokine is a potent platelet agonist highly expressed in atherosclerotic plaques. *Circ Res* 2000; 86: 131–138.

12 Zimmerman GA, McIntyre TM, Prescott SM. Adhesion and signaling in vascular cell–cell interactions. *J Clin Invest* 1997; 100: S3–S5.

13 Lawrence MB, Springer TA. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell* 1991; 65: 859–873.

14 Zimmerman GA, Elstad MR, Lorant DE et al. Platelet-activating factor (PAF): signalling and adhesion in cell-cell interactions. *Adv Exp Med Biol* 1996; 416: 297–304.

15 Newman PJ. The biology of PECAM-1. *J Clin Invest* 1997; 100: S25–S29.

16 Libby P, Geng YJ, Aikawa M et al. Macrophages and atherosclerotic plaque stability. *Curr Opin Lipidol* 1996; 7: 330–335.

17 Bombeli T, Schwartz BR, Harlan JM. Endothelial cells undergoing apoptosis become proadhesive for nonactivated platelets. *Blood* 1999; 93: 3831–3838.

18 Bombeli T, Schwartz BR, Harlan JM. Adhesion of activated platelets to endothelial cells: evidence for a GPIIbIIIa-dependent bridging mechanism and novel roles for endothelial intercellular adhesion molecule 1 (ICAM-1), alphavbeta3 integrin, and GPIbalpha. *J Exp Med* 1998; 187: 329–339.

19 Gawaz M, Neumann FJ, Dickfeld T et al. Vitronectin receptor (alpha(v)beta3) mediates platelet adhesion to the luminal aspect of endothelial cells: implications for reperfusion in acute myocardial infarction. *Circulation* 1997; 96: 1809–1818.

20 Deuel TF, Senior RM, Huang JS, Griffin GL. Chemotaxis of monocytes and neutrophils to platelet-derived growth factor. *J Clin Invest* 1982; 69: 1046–1049.

21 Gorog P, Kovacs IB. Lipid peroxidation by activated platelets:

Cambridge Books Online © Cambridge University Press, 2009

a possible link between thrombosis and atherogenesis. *Atherosclerosis* 1995; 115: 121–128.
22 Mach F, Schonbeck U, Libby P. CD40 signaling in vascular cells: a key role in atherosclerosis? *Atherosclerosis* 1998; 137 Suppl: S89–S95.
23 Mach F, Schonbeck U, Sukhova GK, Atkinson E, Libby P. Reduction of atherosclerosis in mice by inhibition of CD40 signalling. *Nature* 1998; 394: 200–203.
24 Henn V, Slupsky JR, Grafe M et al. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 1998; 391: 591–594.
25 Slupsky JR, Kalbas M, Willuweit A, Henn V, Kroczek RA, Muller-Berghaus G. Activated platelets induce tissue factor expression on human umbilical vein endothelial cells by ligation of CD40. *Thromb Haemost* 1998; 80: 1008–1014.
26 Gawaz M, Neumann FJ, Dickfeld T et al. Activated platelets induce monocyte chemotactic protein-1 secretion and surface expression of intercellular adhesion molecule-1 on endothelial cells. *Circulation* 1998; 98: 1164–1171.
27 Weyrich AS, Elstad MR, McEver RP et al. Activated platelets signal chemokine synthesis by human monocytes. *J Clin Invest* 1996; 97: 1525–1534.
28 Neumann FJ, Marx N, Gawaz M et al. Induction of cytokine expression in leukocytes by binding of thrombin- stimulated platelets. *Circulation* 1997; 95: 2387–2394.
29 Larsen E, Celi A, Gilbert GE et al. PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. *Cell* 1989; 59: 305–312.
30 Furie B, Furie BC. Leukocyte crosstalk at the vascular wall. *Thromb Haemost* 1997; 78: 306–309.
31 Blann AD, Dobrotova M, Kubisz P, McCollum CN. von Willebrand factor, soluble P-selectin, tissue plasminogen activator and plasminogen activator inhibitor in atherosclerosis. *Thromb Haemost* 1995; 74: 626–630.
32 Amirkhosravi A, Alexander M, May K et al. The importance of platelets in the expression of monocyte tissue factor antigen measured by a new whole blood flow cytometric assay. *Thromb Haemost* 1996; 75: 87–95.
33 Celi A, Pellegrini G, Lorenzet R et al. P-selectin induces the expression of tissue factor on monocytes. *Proc Natl Acad Sci USA* 1994; 91: 8767–8771.
34 Lindmark E, Tenno T, Siegbahn A. Role of platelet P-selectin and CD40 ligand in the induction of monocytic tissue factor expression. *Arterioscler Thromb Vasc Biol* 2000; 20: 2322–2328.
35 Giesen PL, Rauch U, Bohrmann B et al. Blood-borne tissue factor: another view of thrombosis. *Proc Natl Acad Sci USA* 1999; 96: 2311–2315.
36 Yeo EL, Sheppard JA, Feuerstein IA. Role of P-selectin and leukocyte activation in polymorphonuclear cell adhesion to surface adherent activated platelets under physiologic shear conditions (an injury vessel wall model). *Blood* 1994; 83: 2498–2507.
37 Kuijper PH, Gallardo Torres HI, Lammers JW, Sixma JJ, Koenderman L, Zwaginga JJ. Platelet and fibrin deposition at the damaged vessel wall: cooperative substrates for neutrophil adhesion under flow conditions. *Blood* 1997; 89: 166–175.
38 Diacovo TG, Puri KD, Warnock RA, Springer TA, von Andrian UH. Platelet-mediated lymphocyte delivery to high endothelial venules. *Science* 1996; 273: 252–255.
39 Theilmeier G, Lenaerts T, Remacle C, Collen D, Vermylen J, Hoylaerts MF. Circulating activated platelets assist THP-1 monocytoid/endothelial cell interaction under shear stress. *Blood* 1999; 94: 2725–2734.
40 Diacovo TG, Roth SJ, Buccola JM, Bainton DF, Springer TA. Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. *Blood* 1996; 88: 146–157.
41 Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993; 362: 801–809.
42 Yla-Herttuala S. Oxidized LDL and atherogenesis. *Ann NY Acad Sci* 1999; 874: 134–137.
43 Frostegard J, Wu R, Giscombe R, Holm G, Lefvert AK, Nilsson J. Induction of T-cell activation by oxidized low density lipoprotein. *Arterioscler Thromb* 1992; 12: 461–467.
44 Salonen JT, Yla-Herttuala S, Yamamoto R et al. Autoantibody against oxidised LDL and progression of carotid atherosclerosis. *Lancet* 1992; 339: 883–887.
45 Yla-Herttuala S, Palinski W, Rosenfeld ME et al. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J Clin Invest* 1989; 84: 1086–1095.
46 Sevitt S. Platelets and foam cells in the evolution of atherosclerosis. Histological and immunohistological studies of human lesions. *Atherosclerosis* 1986; 61: 107–115.
47 Curtiss LK, Black AS, Takagi Y, Plow EF. New mechanism for foam cell generation in atherosclerotic lesions. *J Clin Invest* 1987; 80: 367–373.
48 Curtiss LK, Dyer CA, Banka CL, Black AS. Platelet-mediated foam cell formation in atherosclerosis. *Clin Invest Med* 1990; 13: 189–195.
49 Stary HC. Evolution and progression of atherosclerotic lesions in coronary arteries of children and young adults. *Arteriosclerosis* 1989; 9: I19–I32.
50 Parhami F, Morrow AD, Balucan J et al. Lipid oxidation products have opposite effects on calcifying vascular cell and bone cell differentiation. A possible explanation for the paradox of arterial calcification in osteoporotic patients. *Arterioscler Thromb Vasc Biol* 1997; 17: 680–687.
51 Ward MR, Pasterkamp G, Yeung AC, Borst C. Arterial remodeling. Mechanisms and clinical implications. *Circulation* 2000; 102: 1186–1191.
52 Falk E, Shah PK, Fuster V. Coronary plaque disruption. *Circulation* 1995; 92: 657–671.
53 Schonbeck U, Mach F, Sukhova GK et al. Regulation of matrix metalloproteinase expression in human vascular smooth muscle cells by T lymphocytes: a role for CD40 signaling in plaque rupture? *Circ Res* 1997; 81: 448–454.
54 Toschi V, Gallo R, Lettino M et al. Tissue factor modulates the

Cambridge Books Online © Cambridge University Press, 2009

thrombogenicity of human atherosclerotic plaques. *Circulation* 1997; 95: 594–599.
55 Bjorkerud S, Bjorkerud B. Apoptosis is abundant in human atherosclerotic lesions, especially in inflammatory cells (macrophages and T cells), and may contribute to the accumulation of gruel and plaque instability. *Am J Pathol* 1996; 149: 367–380.
56 Weis JR, Pitas RE, Wilson BD, Rodgers GM. Oxidized low-density lipoprotein increases cultured human endothelial cell tissue factor activity and reduces protein C activation. *FASEB J* 1991; 5: 2459–2465.
57 Farb A, Burke AP, Tang AL et al. Coronary plaque erosion without rupture into a lipid core. A frequent cause of coronary thrombosis in sudden coronary death. *Circulation* 1996; 93: 1354–1363.
58 Fitzgerald DJ, Roy L, Catella F, FitzGerald GA. Platelet activation in unstable coronary disease. *N Engl J Med* 1986; 315: 983–989.
59 Ikeda H, Takajo Y, Ichiki K, Ueno T, Maki S, Noda T et al. Increased soluble form of P-selectin in patients with unstable angina. *Circulation* 1995; 92: 1693–1696.
60 Davies MJ, Thomas AC, Knapman PA, Hangartner JR. Intramyocardial platelet aggregation in patients with unstable angina suffering sudden ischemic cardiac death. *Circulation* 1986; 73: 418–427.
61 Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes (2). *N Engl J Med* 1992; 326: 310–318.
62 Davies MJ, Bland JM, Hangartner JR, Angelini A, Thomas AC. Factors influencing the presence or absence of acute coronary artery thrombi in sudden ischaemic death. *Eur Heart J* 1989; 10: 203–208.
63 Mann J, Davies MJ. Mechanisms of progression in native coronary artery disease: role of healed plaque disruption. *Heart* 1999; 82: 265–268.
64 Badimon L, Badimon JJ, Galvez A, Chesebro JH, Fuster V. Influence of arterial damage and wall shear rate on platelet deposition. Ex vivo study in a swine model. *Arteriosclerosis* 1986; 6: 312–320.
65 Hamsten A, de Faire U, Walldius G et al. Plasminogen activator inhibitor in plasma: risk factor for recurrent myocardial infarction. *Lancet* 1987; 2: 3–9.
66 Heinrich J, Balleisen L, Schulte H, Assmann G, van de LJ. Fibrinogen and factor VII in the prediction of coronary risk. Results from the PROCAM study in healthy men. *Arterioscler Thromb* 1994; 14: 54–59.
67 Redondo M, Watzke HH, Stucki B et al. Coagulation factors II, V, VII, and X, prothrombin gene 20210G→A transition, and factor V Leiden in coronary artery disease: high factor V clotting activity is an independent risk factor for myocardial infarction. *Arterioscler Thromb Vasc Biol* 1999; 19: 1020–1025.
68 Thompson SG, Kienast J, Pyke SD, Haverkate F, van de Loo JC. Hemostatic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. *N Engl J Med* 1995; 332: 635–641.
69 Freedman JE, Ting B, Hankin B, Loscalzo J, Keaney JF, Jr, Vita JA. Impaired platelet production of nitric oxide predicts presence of acute coronary syndromes. *Circulation* 1998; 98: 1481–1486.
70 Freedman JE, Loscalzo J, Barnard MR, Alpert C, Keaney JF, Michelson AD. Nitric oxide released from activated platelets inhibits platelet recruitment. *J Clin Invest* 1997; 100: 350–356.
71 Mehta JL, Chen LY, Kone BC, Mehta P, Turner P. Identification of constitutive and inducible forms of nitric oxide synthase in human platelets. *J Lab Clin Med* 1995; 125: 370–377.
72 Tofler GH, Brezinski D, Schafer AI et al. Concurrent morning increase in platelet aggregability and the risk of myocardial infarction and sudden cardiac death. *N Engl J Med* 1987; 316: 1514–1518.
73 Nievelstein PF, de Groot PG. Interaction of blood platelets with the vessel wall. *Haemostasis* 1988; 18: 342–359.
74 Kuijper PH, Gallardo Torres HI, van der Linden JA et al. Neutrophil adhesion to fibrinogen and fibrin under flow conditions is diminished by activation and L-selectin shedding. *Blood* 1997; 89: 2131–2138.
75 Palabrica T, Lobb R, Furie BC et al. Leukocyte accumulation promoting fibrin deposition is mediated in vivo by P-selectin on adherent platelets. *Nature* 1992; 359: 848–851.
76 Schini-Kerth VB, Fisslthaler B, Obberghen-Schilling E, Busse R. Serotonin stimulates the expression of thrombin receptors in cultured vascular smooth muscle cells. Role of protein kinase C and protein tyrosine kinases. *Circulation* 1996; 93: 2170–2177.
77 Fuster W, Bowie EJ, Lewis JC, Fass DN, Owen CA, Jr., Brown AL. Resistance to arteriosclerosis in pigs with von Willebrand's disease. Spontaneous and high cholesterol diet-induced arteriosclerosis. *J Clin Invest* 1978; 61: 722–730.
78 Adams PC, Lam JY, Badimon L, Chesebro JH, Fuster V. Interactions of platelets and vessel wall in the development of restenosis after coronary angioplasty. *Ann NY Acad Sci* 1987; 516: 605–620.
79 Ip JH, Fuster V, Israel D, Badimon L, Badimon J, Chesebro JH. The role of platelets, thrombin and hyperplasia in restenosis after coronary angioplasty. *J Am Coll Cardiol* 1991; 17: 77B–88B.
80 Fridman RJ, Stemerman MB, Wenz B et al. The effect of thrombocytopenia on experimental atherosclerotic lesion formation in rabbits. Smooth muscle cell proliferation and re-endothelialization. *J Clin Invest* 1977; 60: 1191–1201.
81 Matsuno H, Stassen JM, Vermylen J, Deckmyn H. Inhibition of integrin function by a cyclic RGD-containing peptide prevents neointima formation. *Circulation* 1994; 90: 2203–2206.
82 Kimura T, Nobuyoshi M. Remodelling and restenosis: intravascular ultrasound studies. *Semin Interv Cardiol* 1997; 2: 159–166.
83 Bertrand ME, Rupprecht HJ, Urban P, Gershlick AH, Investigators f. Double-blind study of the safety of clopidogrel with and without a loading dose in combination with

Cambridge Books Online © Cambridge University Press, 2009

aspirin compared with ticlopidine in combination with aspirin after coronary stenting: the clopidogrel aspirin stent international cooperative study (CLASSICS). *Circulation* 2000; 102: 624–629.

84 Thaulow E, Erikssen J, Sandvik L, Stormorken H, Cohn PF. Blood platelet count and function are related to total and cardiovascular death in apparently healthy men. *Circulation* 1991; 84: 613–617.

85 Elwood PC, Beswick AD, Sharp DS, Yarnell JW, Rogers S, Renaud S. Whole blood impedance platelet aggregometry and ischemic heart disease. The Caerphilly Collaborative Heart Disease Study. *Arteriosclerosis* 1990; 10: 1032–1036.

86 Elwood PC, Renaud S, Sharp DS, Beswick AD, O'Brien JR, Yarnell JW. Ischemic heart disease and platelet aggregation. The Caerphilly Collaborative Heart Disease Study. *Circulation* 1991; 83: 38–44.

87 Elwood PC, Renaud S, Beswick AD, O'Brien JR, Sweetnam PM. Platelet aggregation and incident ischaemic heart disease in the Caerphilly cohort. *Heart* 1998; 80: 578–582.

88 Trip MD, Cats VM, van Capelle FJ, Vreeken J. Platelet hyperreactivity and prognosis in survivors of myocardial infarction. *N Engl J Med* 1990; 322: 1549–1554.

89 Martin JF, Bath PM, Burr ML. Influence of platelet size on outcome after myocardial infarction. *Lancet* 1991; 338: 1409–1411.

90 Hennekens CH, Buring JE. Aspirin in the primary prevention of cardiovascular disease. *Cardiol Clin* 1994; 12: 443–450.

91 Final report on the aspirin component of the ongoing Physicians' Health Study. Steering Committee of the Physicians' Health Study Research Group. *N Engl J Med* 1989; 321: 129–135.

92 Renaud S, de Lorgeril M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* 1992; 339: 1523–1526.

93 Stone NJ. The Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardio (GISSI)-Prevenzione Trial on fish oil and vitamin E supplementation in myocardial infarction survivors. *Curr Cardiol Rep* 2000; 2: 445–451.

94 Zhu BQ, Sievers RE, Sun YP, Morse-Fisher N, Parmley WW, Wolfe CL. Is the reduction of myocardial infarct size by dietary fish oil the result of altered platelet function? *Am Heart J* 1994; 127: 744–755.

95 Burr ML, Fehily AM, Gilbert JF et al. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART) [see comments]. *Lancet* 1989; ii: 757–761.

96 Blann AD, Lip GY, Beevers DG, McCollum CN. Soluble P-selectin in atherosclerosis: a comparison with endothelial cell and platelet markers. *Thromb Haemost* 1997; 77: 1077–1080.

97 Gawaz M, Neumann FJ, Schomig A. Evaluation of platelet membrane glycoproteins in coronary artery disease: consequences for diagnosis and therapy. *Circulation* 1999; 99: e1–11.

98 Patrono C, FitzGerald GA. Isoprostanes: potential markers of oxidant stress in atherothrombotic disease. *Arterioscler Thromb Vasc Biol* 1997; 17: 2309–2315.

99 Morrow JD, Awad JA, Boss HJ, Blair IA, Roberts LJ. Non-cyclooxygenase-derived prostanoids (F2-isoprostanes) are formed in situ on phospholipids. *Proc Natl Acad Sci USA* 1992; 89: 10721–10725.

100 Davi G, Alessandrini P, Mezzetti A et al. In vivo formation of 8–Epi-prostaglandin F2 alpha is increased in hypercholesterolemia. *Arterioscler Thromb Vasc Biol* 1997; 17: 3230–3235.

101 Lynch SM, Morrow JD, Roberts LJ, Frei B. Formation of non-cyclooxygenase-derived prostanoids (F2-isoprostanes) in plasma and low density lipoprotein exposed to oxidative stress in vitro. *J Clin Invest* 1994; 93: 998–1004.

102 Davi G, Ciabattoni G, Consoli A et al. In vivo formation of 8-iso-prostaglandin f2alpha and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation. *Circulation* 1999; 99: 224–229.

103 Reilly M, Delanty N, Lawson JA, FitzGerald GA. Modulation of oxidant stress in vivo in chronic cigarette smokers. *Circulation* 1996; 94: 19–25.

104 Nityanand S, Pande I, Bajpai VK, Singh L, Chandra M, Singh BN. Platelets in essential hypertension. *Thromb Res* 1993; 72: 447–454.

105 Yamada M, Omata K, Abe F, Ito S, Abe K. Changes in prostacyclin, thromboxane A2 and F2-isoprostanes, and influence of eicosapentaenoic acid and antiplatelet agents in patients with hypertension and hyperlipidemia. *Immunopharmacology* 1999; 44: 193–198.

106 Welch GN, Loscalzo J. Homocysteine and atherothrombosis. *N Engl J Med* 1998; 338: 1042–1050.

107 Di Minno G, Davi G, Margaglione M et al. Abnormally high thromboxane biosynthesis in homozygous homocystinuria. Evidence for platelet involvement and probucol-sensitive mechanism. *J Clin Invest* 1993; 92: 1400–1406.

108 Dardik R, Varon D, Tamarin I et al. Homocysteine and oxidized low density lipoprotein enhanced platelet adhesion to endothelial cells under flow conditions: distinct mechanisms of thrombogenic modulation. *Thromb Haemost* 2000; 83: 338–344.

109 Levine PH. An acute effect of cigarette smoking on platelet function. A possible link between smoking and arterial thrombosis. *Circulation* 1973; 48: 619–623.

110 Newby DE, Wright RA, Labinjoh C et al. Endothelial dysfunction, impaired endogenous fibrinolysis, and cigarette smoking: a mechanism for arterial thrombosis and myocardial infarction. *Circulation* 1999; 99: 1411–1415.

111 Matetzky S, Tani S, Kangavari S et al. Smoking increases tissue factor expression in atherosclerotic plaques: implications for plaque thrombogenicity. *Circulation* 2000; 102: 602–604.

112 Kwaan HC, Colwell JA, Cruz S, Suwanwela N, Dobbie JG. Increased platelet aggregation in diabetes mellitus. *J Lab Clin Med* 1972; 80: 236–246.

113 Tschoepe D, Roesen P, Kaufmann L et al. Evidence for abnormal platelet glycoprotein expression in diabetes mellitus. *Eur J Clin Invest* 1990; 20: 166–170.

Cambridge Books Online © Cambridge University Press, 2009

114 Tschoepe D, Roesen P, Esser J et al. Large platelets circulate in an activated state in diabetes mellitus. *Semin Thromb Hemost* 1991; 17: 433–438.
115 Tschoepe D, Driesch E, Schwippert B, Lampeter EF. Activated platelets in subjects at increased risk of IDDM. DENIS Study Group. Deutsche Nikotinamid Interventionsstudie. *Diabetologia* 1997; 40: 573–577.
116 McCance DR, Dyer DG, Dunn JA et al. Maillard reaction products and their relation to complications in insulin-dependent diabetes mellitus. *J Clin Invest* 1993; 91: 2470–2478.
117 Yamagishi S, Yamamoto Y, Harada S, Hsu CC, Yamamoto H. Advanced glycosylation end products stimulate the growth but inhibit the prostacyclin-producing ability of endothelial cells through interactions with their receptors. *FEBS Lett* 1996; 384: 103–106.
118 Hangaishi M, Taguchi J, Miyata T et al. Increased aggregation of human platelets produced by advanced glycation end products in vitro. *Biochem Biophys Res Commun* 1998; 248: 285–292.
119 Khechai F, Ollivier V, Bridey F, Amar M, Hakim J, de Prost D. Effect of advanced glycation end product-modified albumin on tissue factor expression by monocytes. Role of oxidant stress and protein tyrosine kinase activation. *Arterioscler Thromb Vasc Biol* 1997; 17: 2885–2890.
120 Yan SD, Schmidt AM, Anderson GM et al. Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. *J Biol Chem* 1994; 269: 9889–9897.
121 Gopaul NK, Anggard EE, Mallet AI, Betteridge DJ, Wolff SP, Nourooz-Zadeh J. Plasma 8-epi-PGF2 alpha levels are elevated in individuals with non- insulin dependent diabetes mellitus. *FEBS Lett* 1995; 368: 225–229.
122 Kodama M, Yamasaki Y, Sakamoto K et al. Antiplatelet drugs attenuate progression of carotid intima-media thickness in subjects with type 2 diabetes. *Thromb Res* 2000; 97: 239–245.
123 Surya II, Akkerman JW. The influence of lipoproteins on blood platelets. *Am Heart J* 1993; 125: 272–275.
124 Davi G, Romano M, Mezzetti A et al. Increased levels of soluble P-selectin in hypercholesterolemic patients. *Circulation* 1998; 97: 953–957.
125 Davi G, Averna M, Catalano I et al. Increased thromboxane biosynthesis in type IIa hypercholesterolemia. *Circulation* 1992; 85: 1792–1798.
126 Aviram M, Brook GJ. Platelet interaction with high and low density proteins. *Atherosclerosis* 1983; 46: 259–268.
127 Ardlie NG, Selley ML, Simons LA. Platelet activation by oxidatively modified low density lipoproteins. *Atherosclerosis* 1989; 76: 117–124.
128 Bochkov VN, Matchin YG, Fuki IV, Lyakishev AA, Tkachuk VA. Platelets in patients with homozygous familial hypercholesterolemia are sensitive to Ca^{2+}-mobilizing activity of low density lipoproteins. *Atherosclerosis* 1992; 96: 119–124.
129 Andrews HE, Aitken JW, Hassall DG, Skinner VO, Bruckdorfer KR. Intracellular mechanisms in the activation of human platelets by low-density lipoproteins. *Biochem J* 1987; 242: 559–564.
130 Dunn RC, Schachter M, Miles CM et al. Low-density lipoproteins increase intracellular calcium in aequorin- loaded platelets. *FEBS Lett* 1988; 238: 357–360.
131 Hayek T, Oiknine J, Brook JG, Aviram M. Increased plasma and lipoprotein lipid peroxidation in apo E-deficient mice. *Biochem Biophys Res Commun* 1994; 201: 1567–1574.
132 Holvoet P, Vanhaecke J, Janssens S, Van de WF, Collen D. Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. *Circulation* 1998; 98: 1487–1494.
133 Weidtmann A, Scheithe R, Hrboticky N, Pietsch A, Lorenz R, Siess W. Mildly oxidized LDL induces platelet aggregation through activation of phospholipase A2. *Arterioscler Thromb Vasc Biol* 1995; 15: 1131–1138.
134 Murohara T, Scalia R, Lefer AM. Lysophosphatidylcholine promotes P-selectin expression in platelets and endothelial cells. Possible involvement of protein kinase C activation and its inhibition by nitric oxide donors. *Circ Res* 1996; 78: 780–789.
135 Zhao B, Dierichs R, Harrach-Ruprecht B, Winterhorff H. Oxidized LDL induces serotonin release from blood platelets. *Am J Hematol* 1995; 48: 285–287.
136 Siess W, Zangl KJ, Essler M et al. Lysophosphatidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions. *Proc Natl Acad Sci USA* 1999; 96: 6931–6936.
137 Essler M, Retzer M, Bauer M, Zangl KJ, Tigyi G, Siess W. Stimulation of platelets and endothelial cells by mildly oxidized LDL proceeds through activation of lysophosphatidic acid receptors and the Rho/Rho-kinase pathway. Inhibition by lovastatin. *Ann NY Acad Sci* 2000; 905: 282–286.
138 Chen LY, Mehta JL. Variable effects of L-arginine analogs on L-arginine-nitric oxide pathway in human neutrophils and platelets may relate to different nitric oxide synthase isoforms. *J Pharmacol Exp Ther* 1996; 276: 253–257.
139 Beswick AD, Fehily AM, Sharp DS, Renaud S, Giddings J. Long-term diet modification and platelet activity. *J Intern Med* 1991; 229: 511–515.
140 Wolf A, Zalpour C, Theilmeier G et al. Dietary L-arginine supplementation normalizes platelet aggregation in hypercholesterolemic humans. *J Am Coll Cardiol* 1997; 29: 479–485.
141 Diodati J, Theroux P, Latour JG, Lacoste L, Lam JY, Waters D. Effects of nitroglycerin at therapeutic doses on platelet aggregation in unstable angina pectoris and acute myocardial infarction. *Am J Cardiol* 1990; 66: 683–688.
142 Gotto AM, Jr. Lipid-lowering trials: what have they taught us about morbidity and mortality? *Cardiology* 1996; 87: 453–457.
143 Kockx MM, De Meyer GRY, Buyssens N, Knaapen MWM, Bult H, Herman AG. Cell composition, replication, and apoptosis in atherosclerotic plaques after 6 months of cholesterol withdrawal. *Circ Res* 1998; 83: 378–387.
144 Aikawa M, Voglic SJ, Sugiyama S et al. Dietary lipid lowering reduces tissue factor expression in rabbit atheroma. *Circulation* 1999; 100: 1215–1222.

Cambridge Books Online © Cambridge University Press, 2009

145 Aviram M, Hussein O, Rosenblat M, Schlezinger S, Hayek T, Keidar S. Interactions of platelets, macrophages, and lipoproteins in hypercholesterolemia: antiatherogenic effects of HMG-CoA reductase inhibitor therapy. *J Cardiovasc Pharmacol* 1998; 31: 39–45.
146 Notarbartolo A, Davi G, Averna M et al. Inhibition of thromboxane biosynthesis and platelet function by simvastatin in type IIa hypercholesterolemia. *Arterioscler Thromb Vasc Biol* 1995; 15: 247–251.
147 Hochgraf E, Levy Y, Aviram M, Brook JG, Cogan U. Lovastatin decreases plasma and platelet cholesterol levels and normalizes elevated platelet fluidity and aggregation in hypercholesterolemic patients. *Metabolism* 1994; 43: 11–17.
148 Alfon J, Royo T, Garcia-Moll X, Badimon L. Platelet deposition on eroded vessel walls at a stenotic shear rate is inhibited by lipid-lowering treatment with atorvastatin. *Arterioscler Thromb Vasc Biol* 1999; 19: 1812–1817.
149 Diaz MN, Frei B, Vita JA, Keaney JF, Jr. Antioxidants and atherosclerotic heart disease. *N Engl J Med* 1997; 337: 408–416.
150 Freedman JE, Farhat JH, Loscalzo J, Keaney JF, Jr. Alpha-tocopherol inhibits aggregation of human platelets by a protein kinase C-dependent mechanism. *Circulation* 1996; 94: 2434–2440.
151 Yusuf S, Dagenais G, Pogue J, Bosch J, Sleight P. Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators [see comments]. *N Engl J Med* 2000; 342: 154–160.
152 Virtamo J, Rapola JM, Ripatti S et al. Effect of vitamin E and beta carotene on the incidence of primary nonfatal myocardial infarction and fatal coronary heart disease. *Arch Intern Med* 1998; 158: 668–675.
153 Christen WG, Gaziano JM, Hennekens CH. Design of Physicians' Health Study II – a randomized trial of beta-carotene, vitamins E and C, and multivitamins, in prevention of cancer, cardiovascular disease, and eye disease, and review of results of completed trials. *Ann Epidemiol* 2000; 10: 125–134.
154 Stephens NG, Parsons A, Schofield PM, Kelly F, Cheeseman K, Mitchinson MJ. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet* 1996; 347: 781–786.
155 Shih DM, Xia YR, Wang X et al. Combined serum paraoxonase Knockout/Apolipoprotein E knockout mice exhibit increased lipoprotein oxidation and atherosclerosis. *J Biol Chem* 2000; 275: 17527–17535.
156 Theilmeier G, De Geest B, Van Veldhoven PP et al. HDL-associated PAF-AH reduces endothelial adhesiveness in apoE-/- mice. *FASEB J* 2000; 14: 2032–2039.
157 Hassall DG, Forrest LA, Bruckdorfer KR et al. Influence of plasma lipoproteins on platelet aggregation in a normal male population. *Arteriosclerosis* 1983; 3: 332–338.
158 Aviram M, Brook JG. Platelet interaction with high and low density lipoproteins. *Atherosclerosis* 1983; 46: 259–268.
159 Yui Y, Aoyama T, Morishita H, Takahashi M, Takatsu Y, Kawai C. Serum prostacyclin stabilizing factor is identical to apolipoprotein A- I (Apo A-I). A novel function of Apo A-I. *J Clin Invest* 1988; 82: 803–807.
160 Li D, Weng S, Yang B et al. Inhibition of arterial thrombus formation by ApoA1 Milano. *Arterioscler Thromb Vasc Biol* 1999; 19: 378–383.
161 Schildkraut JM, Myers RH, Cupples LA, Kiely DK, Kannel WB. Coronary risk associated with age and sex of parental heart disease in the Framingham Study. *Am J Cardiol* 1989; 64: 555–559.
162 Weiss EJ, Bray PF, Tayback M et al. A polymorphism of a platelet glycoprotein receptor as an inherited risk factor for coronary thrombosis. *N Engl J Med* 1996; 334: 1090–1094.
163 Ridker PM, Hennekens CH, Schmitz C, Stampfer MJ, Lindpaintner K. PIA1/A2 polymorphism of platelet glycoprotein IIIa and risks of myocardial infarction, stroke, and venous thrombosis. *Lancet* 1997; 349: 385–388.
164 Faraci FM, Sigmund CD. Vascular biology in genetically altered mice: smaller vessels, bigger insight. *Circ Res* 1999; 85: 1214–1225.
165 Davies MJ, Richardson PD, Woolf N, Katz DR, Mann J. Risk of thrombosis in human atherosclerotic plaques: role of extracellular lipid, macrophage, and smooth muscle cell content. *Br Heart J* 1993; 69: 377–381.
166 Toussaint JF, LaMuraglia GM, Southern JF, Fuster V, Kantor HL. Magnetic resonance images of lipid, fibrous, calcified, hemorrhagic, and thrombotic components of human atherosclerosis in vivo. *Circulation* 1996; 94: 932–938.
167 Kuijper PH, Gallardo Torres HI, van der Linden JA et al. Platelet-dependent primary hemostasis promotes sele. *Blood* 1996; 87: 3271–3281.
168 Rubenstein MD, Wall RT, Baim DS, Harrison DC. Platelet activation in clinical coronary artery disease and spasm. *Am Heart J* 1981; 102: 363–367.
169 Dalal JJ, Sheridan DJ, Bloom AL, Henderson AH. Platelet aggregates and coronary spasm [letter]. *Lancet* 1980; ii: 1146–1147.
170 Zhao B, Rickert CH, Filler TJ, Liu B, Verhallen PF, Dierichs R. Adhesion of washed blood platelets in vitro is advanced, accelerated, and enlarged by oxidized low-density lipoprotein. *Am J Hematol* 1995; 49: 177–182.
171 Paolucci S, Buttinelli C, Lucignani G, Prencipe M. Platelet in vivo aggregation and aging. *Monogr Neural Sci* 1984; 11: 240–242.
172 Renaud SC, Beswick AD, Fehily AM, Sharp DS, Elwood PC. Alcohol and platelet aggregation: the Caerphilly Prospective Heart Disease Study. *Am J Clin Nutr* 1992; 55: 1012–1017.

Cambridge Books Online © Cambridge University Press, 2009

Platelet involvement in venous thrombosis and pulmonary embolism

Andrew D. Blann and Gregory Y.H. Lip

Haemostasis, Thrombosis and Vascular Biology Unit, University Department of Medicine, City Hospital, Birmingham UK

Introduction

Since their discovery in 1842[1], platelets have commanded an increasingly prominent position in the pathophysiology of thrombosis, although this has been almost exclusively in arterial disease. However, Virchow was most likely the first to link changes in the blood (that we would today interpret as rheology, vascular function, and the soluble and cellular constituents of the blood) with venous thrombosis[2].

There are three major lines of approach that we can take in order to test the hypothesis that platelets are intimately involved in the development of DVT and PE. The pharmacological approach considers clinical trials of antiplatelet drugs (such as aspirin) in well-defined groups of high-risk patients that demonstrate a reduction in the rate and/or risk of thrombosis. The laboratory approach can be used to demonstrate changes in platelet function ex vivo or in levels of platelet-specific markers (such as beta thromboglobulin) in the plasma of those at risk of, with, or having suffered, a thrombotic event. More recently, radionucleotide and other imaging with labelled platelets, antibodies, probes or other agents provide additional evidence of the role of platelets in DVT and PE. Whilst there are considerable animal data on the subject of venous thromboembolism, this overview will be confined to human studies.

Clinical trials

The most commonly used and most successful antiplatelet drug is aspirin, which is well established as secondary prevention in vascular (arterial) disease. A recent report that aspirin inhibits smooth muscle cell proliferation may also be relevant in arterial diseases, but seems to be less important[3].

The rationale for testing the efficacy of aspirin in venous thrombosis followed directly from reports of its ability, for example, to inhibit platelet aggregatory function in vitro[4]. Perhaps the earliest clinical trial of aspirin in the prevention of postoperative DVT[5], where the frequency of the single end point of DVT, as defined by uptake of ^{125}I-fibrinogen, did not differ between those 150 subjects on placebo compared to 153 subjects who took 600 mg aspirin once before their operation and daily for the next 4 days. Lack of efficacy may have been due to the confounding inclusion of 44 subjects with a malignancy and 15 with a past history of thromboembolism, and to the short duration of treatment. Morris and Mitchell[6] were also unable to find a protective effect of aspirin, whilst Harris et al.[7] found a positive effect in men alone.

Another small study, recruiting 120 patients due for hip replacement, found that aspirin (1000 or 250 mg daily) reduced the frequency of postsurgical DVT from 30% to 3.3%[8]. Similarly, Salzman et al.[9] who defined DVT and/or PE on clinical grounds alone, found that aspirin reduced the frequency of venous thrombosis. These early studies therefore, provided conflicting results, and most were underpowered to show a clear benefit or otherwise from aspirin.

The antiplatelet Trialists Collaboration

A major landmark was the meta-analyses of the Antiplatelet Trialists Collaboration[10], which was an overview of some 9000 randomized subjects undergoing general and orthopaedic surgery, and some high-risk medical patients. This showed a clear reduction in DVT (by 39%) and PE (by 64%) that was due to an effect of aspirin (Fig. 49.1). Among the subanalyses was the finding that a combination of aspirin and dipyridamole was more

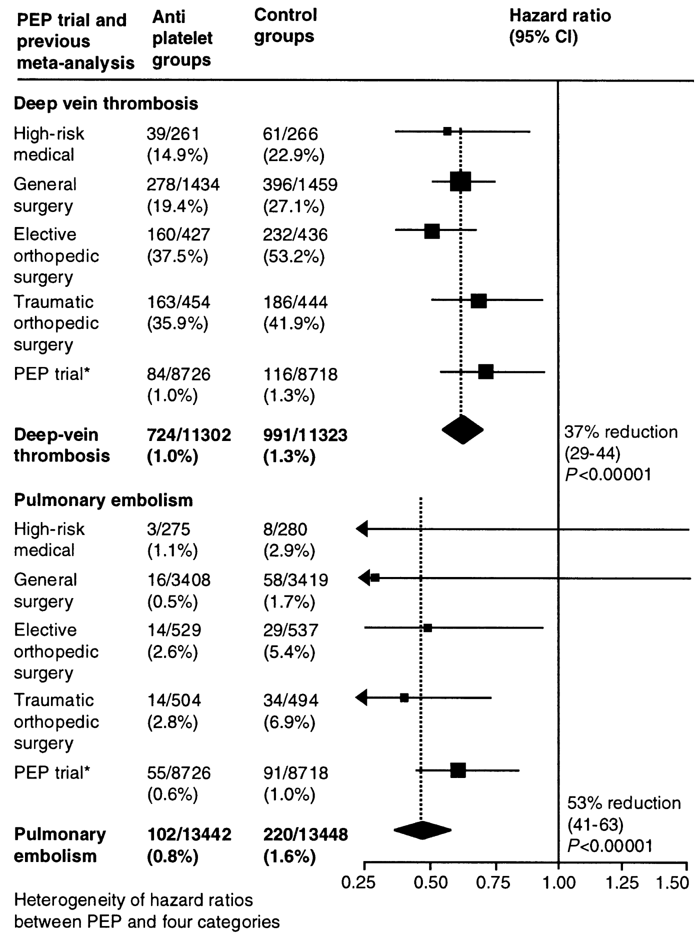


Fig. 49.1. Proportional effects of antiplatelet therapy on venous thromboembolism in previous meta-analysis and the PEP trial. (This is Fig. 5[10], with permission from Dr A. Rodgers.)

effective in reducing the risk of a DVT than the use of aspirin alone (15 events from 109 subjects vs. 33 events from 109 subjects, respectively, $2P=0.001$). Other subanalyses, in both DVT and PE, also demonstrated that the use of hydroxychloroquine was superior to placebo in reducing thrombosis. However, Ticlopidine failed to reduce the risk of either type of thrombosis.

Despite the apparent power of this analysis, some concerns about the conclusions and recommendations of this meta-analysis were raised, for example, that the absolute benefits of aspirin seem to be greater for those at higher risk – for example, those undergoing orthopedic surgery[11]. These issues were subsequently responded to[12], where the increased risk of non-fatal 'major' bleeds ($P=0.04$) and the combination of reoperation, hematoma, or infection due to bleed ($P=0.003$), apparently due to aspirin, were emphasized. However, several of these concerns have been recently answered in a further trial, the Pulmonary Embolism Prevention (PEP) Trial[13].

The Pulmonary Embolism Prevention (PEP) Trial

The Pulmonary Embolism Prevention (PEP) Trial[13] was a randomized, double blind international multicentre trial of the effects of aspirin, in 13 356 patients at risk of thrombosis by virtue of, for example, hip fracture or elective arthroplasty, who were randomized to aspirin 160 mg daily, or placebo, for 35 days: endpoints were mortality and in-hospital morbidity up to Day 35. Principal results (Fig. 49.1) demonstrated a 37% overall reduction in DVT, and a 53% reduction in PE (both $P<0.00001$), with significant reductions in both end points in various subgroups, such as 'high-risk' medical, or following elective, general or traumatic orthopedic surgery. There were no significant differences in bleeding episodes between the groups. The impressive statistical power and thorough methodology of this study provides unequivocal proof of the benefit of aspirin under these conditions.

Non-aspirin interventions

There are isolated reports of the effectiveness (or not) of other antiplatelet agents in the prevention of venous thrombosis. For example, aurin tricarboxylic acid inhibits ristocetin-induced human platelet aggregation in vitro and reduces venous thrombus formation and disseminated intravascular coagulation in in vivo animal models[14]. However, despite this promising data, this agent is rarely used in human disease.

Kakkar et al.[15] reported a synergistic effect of heparin and dihydroergotamine in reducing platelet activity, as judged by aggregation, morphology and changes in plasma levels of the platelet marker, beta thromboglobulin. These observations suggest that heparin may have some effects beyond the enhancement of the anticoagulant activity of antithrombin III. Although Alfaro et al. showed that a combination of heparin and dihydroergotamine reduced the frequency of DVT from 30% to 16.6%, this difference was not statistically significant, possibly due to the small patient numbers studied[8]. Salzman et al.[9] found that warfarin and dextran, like aspirin, also reduced the risk of venous thrombosis, but the reduced frequency of thrombosis in patients taking dipyridamole by one-third (i.e. 26% compared to a control rate of 39%) did not reach statistical significance.

Cambridge Books Online © Cambridge University Press, 2009

Laboratory markers

Although platelet hyperaggregability following DVT has been reported[16], research in this area has focussed on three platelet-specific markers found in the plasma. Platelet factor 4 (PF4) was first described in the late 1960s, beta thromboglobulin a few years later[17–19], whilst soluble P selectin became widely reported in the 1990s[20,21].

Platelet factor 4

PF4, a heparin binding protein first described by Niewiarowski and Thomas[17], exists at a concentration of 11–12 μg per 10^9 platelets. It is a heat stable, low molecular weight (7.8 kDa) protein of 70 amino acids. However, it circulates as a tetramer, which increases the molecular weight of the molecule to about 30 kDa. These complexed tetramers are also associated with two high molecular weight proteoglycan carriers.

The presence of PF4 binding sites on the platelet surface implies that PF4 also has the ability to regulate cell–cell interactions between platelets. With heparin binding sites within a lysine-rich region, it can interact with heparin-like glycosaminoglycans on endothelial cells, thus promoting a procoagulant effect[22,23]. Consequently, the presence of this anticoagulant in the blood stream up-regulates the effective presence of PF4 by up to a factor of 20-fold[24]. This could be a major cause of measurement error as many diseases that have acute thrombotic symptoms are frequently treated in this way. Crucially, aspirin causes a reduction in the PF4 concentration[8,25,26], implying a relationship with the cyclooxygenase/prostacyclin pathways.

Beta thromboglobulin

This molecule consists of four identical subunits, each of 81 amino acid residues having a molecular weight of 8.8 kDa, and as such contributes up to 10% of the α-granules' contents, making it the most abundant platelet specific protein. It is present exclusively in megakaryocytes and in the α-granules of platelets and is present in a range of 8–24 μg per 10^9 platelets. The platelet concentration can reach 260 000 times that of the plasma concentration, making it easy to distinguish when activation has occurred. With a primary structure similar to PF4, β-thromboglobulin is released under the influence of known platelet activators such as ADP, collagen, immune complexes and thrombin, and has a plasma half-life of 100 minutes[15–17,23,26]. It is also excreted in the urine[27], and some researchers[28] advocate measurement in the latter, as it is less prone to artefactual errors, due to collection and processing of venous blood. Others advocate the use of simultaneous plasma PF4 and β-thromboglobulin measurements for a more balanced picture[29]. However, as β-thromboglobulin is cleared by the kidney, the patients' renal function needs to be taken into consideration. It has been suggested that high β-thromboglobulin levels could simply reflect renal impairment rather than the activation of platelets *per se*[30,31]. As is the case for PF4, aspirin reduces levels of this marker[8,25,26].

Soluble P selectin (CD62P)

The adhesion molecule P-selectin was first identified on the surface of activated platelets[32], and subsequently, as part of the membrane of the Weibel–Palade bodies in endothelial cells[33]. P-selectin is now known to be a component of the alpha granule membrane and, when expressed externally due to activation, extends 40 nm out from the membrane surface. Consequently, P-selectin has the capacity to mediate interactions between the endothelium, platelets and leukocytes, possibly by phosphorylation of histidine residues on the cytoplasmic tail of the molecule. The finding of a soluble form (i.e. soluble P-selectin) in the plasma implies a physiological shedding and/or a pathological cleavage of the membrane form[19,20].

Increased levels of this adhesion molecule in the plasma of patients with thrombotic disorders and atherosclerosis (i.e. soluble P-selectin) provides support for the hypothesis that such levels reflect platelet activation[19–21], a concept gaining acceptance[34–36]. In contrast to its effects on PF4 and β-thromboglobulin, aspirin appears to have no effect on levels of soluble P-selectin[35–37]. However, this has recently been challenged in a small, preliminary, cross-sectional report that also found lower membrane P-selectin (defined by flow cytometry) in subjects with an acute myocardial infarction who were already taking aspirin[38]. Unlike β-thromboglobulin, soluble P-selectin is not found in normal urine[28,37]. Although, as with PF4 and β-thromboglobulin, the literature on soluble P-selectin is dominated by data on arterial thrombosis, isolated reports in venous thrombosis exist.

Cross-sectional studies

Several simple case/control studies, often despite small numbers of patients, have reported raised levels of these markers in patients with concurrent venous disease. One of the first found raised levels of β-thromboglobulin in 24 patients presenting with a DVT compared to 16 healthy controls[39]. Cella et al.[40] and Hirose and Inada[41] confirmed this result in larger numbers, whilst Farrell et al.[42] and van

Cambridge Books Online © Cambridge University Press, 2009

Hulsteijn et al.[43] found raised levels in pulmonary embolus. The latter also reported that heparin and warfarin failed to reduce β-thromboglobulin. Sakaguchi et al. reported higher β-thromboglobulin in patients with multiple or recurrent DVT compared to those with a single episode of DVT[44]. However, others have not found significant differences in levels of β-thromboglobulin[45].

Therefore, with a single caveat[45] raised levels of β-thromboglobulin seem to be present in those with a current, acute thrombosis[39–44]. However, levels are not raised in those with a past DVT (>3 weeks)[40]. Van Hulsteijn found that increased β-thromboglobulin was still present in about 50% of patients 3 months after suffering a venous thrombosis: levels returned to normal in all patients after 1 year[46].

Smith and colleagues reported moderately increased ($P = 0.018$) levels of soluble P-selectin in patients with a current, established DVT (either spontaneous or after total hip replacement), compared to controls[47]. Our own study[48] also found moderately ($P = 0.011$) increased levels of soluble P-selectin in the plasma of 89 patients, but a median of 18 months after they had suffered a DVT, and so were considered free of active disease, compared to 126 controls. This effect was independent of smoking (Fig. 49.2). However, the wide spread of this data underlines the lack of specificity of many plasma markers, and thus, some difficulty in interpretation.

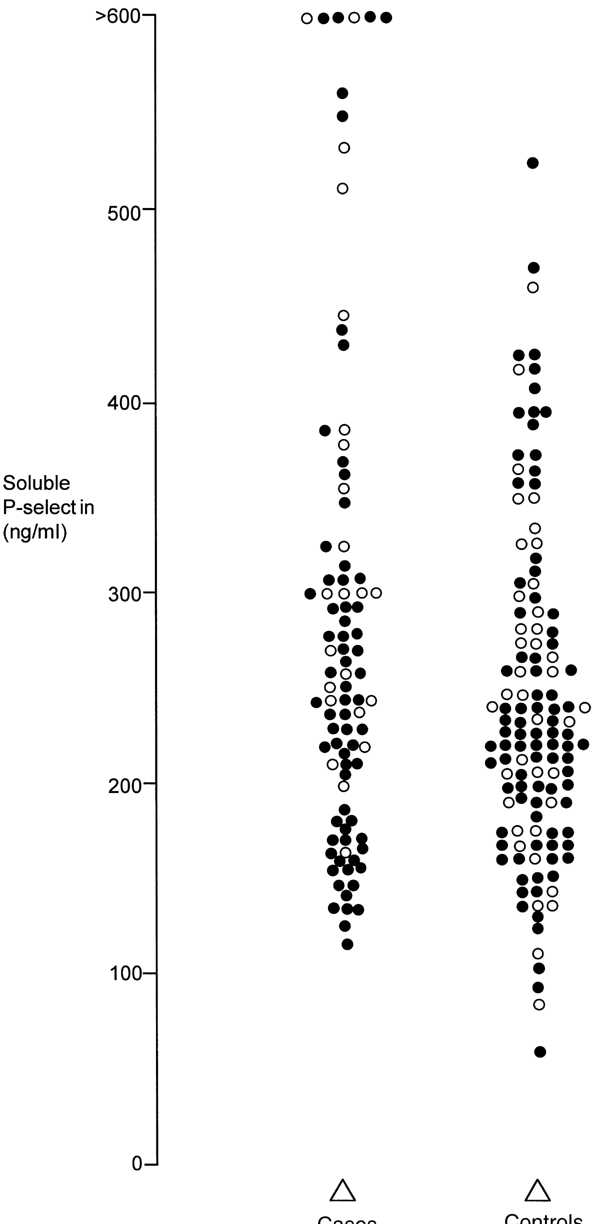


Fig. 49.2. Levels of soluble P selectin in subjects who have had a DVT compared with subjects who have not had a DVT. Open circles: smokers, closed circles: non-smokers[52].

Do platelet markers predict DVT?

The most valuable data suggesting a causal association between platelet activation and venous thromboembolism would be that which predicts the development of a DVT or a PE. As surgery is an established risk factor for thrombosis, several groups have used serial platelet markers in an attempt to do just this. Although Lane et al.[49] and Paramo and Rocha[50] both noted rising levels of β-thromboglobulin following surgery, levels failed to predict those who suffered a DVT. However, Owen et al.[51] failed to find any change in β-thromboglobulin or PF4 after craniotomy, and could also not predict those 18 from 32 developing a DVT. Despite this, it seems that urinary β-thromboglobulin may be superior, as rising levels in the postoperative period did predict those developing a DVT[52]. Similarly, Douglas et al.[53] found that plasma levels were significantly increased on the first postoperative day in 14 patients who developed a DVT after gastrointestinal surgery compared to those 18 patients who did not.

One possible reason for the failure of these studies to predict the thrombosis development is that venepuncture and/or DVT screening may have been at an inopportune window. Another may be inadequate numbers, bringing low statistical power when compared with the huge number of patients involved in the larger clinical trials[8,12]. Both platelet factor 4 and β-thromboglobulin are also prone to the artefact of ex-vivo release from the platelet α-granules once the blood has been drawn, possibly giving rise to spurious data, although this effect can be reduced

Cambridge Books Online © Cambridge University Press, 2009

with a special platelet-friendly cocktail[17,25–27,29]. This latter problem may not be relevant for non-α-granule markers such as those derived from the membrane system of the platelet. However, there are, as yet, no studies reporting the value of markers such as soluble P-selectin or soluble gpV in predicting venous thrombosis. The two studies described[47,48] cannot answer the important question of whether or not raised levels predict venous thrombosis. However, the fact that soluble P-selectin was still raised 18 months after a DVT[48], contrasts with the report of normal values of β-thromboglobulin 1 year after the DVT[46]. The clinical corollary of these differences is uncertain, and the changes may simply reflect measurement of different aspects of platelet physiology.

Platelet imaging

Among the earliest detection methods for DVT are radiolabelled fibrinogen and systematic venography[6–8,10,16,54]. However, due to advances in nuclear medicine, other techniques have become available. For example, Sundrehagen et al.[55] radiolabelled platelets with technetium-99m and used it to detect lung embolism and DVT. Morimoto et al.[56] recently used ^{111}indium to label autologous platelets, and then detected arterial and venous thrombosis. However, long before these studies, Wu et al.[16] used ^{51}chromium labelling to show a correlation between platelet hyperactivity and shortened survival in DVT patients.

Other techniques, possibly more sensitive, have been used that take advantage of various specific platelet surface molecules, as opposed to the entire cell. For example, Muto et al.[57] used a technetium-labelled synthetic peptide of 26 amino acids (P280) with affinity to gpIIb/IIIa (the platelet fibrinogen receptor) in scintigraphy to localize DVTs, and compared it with contrast venography, finding excellent sensitivity, specificity and agreement[58]. The gpIIb/IIIa complex has been the target of several other approaches using different peptides[59,60]. These studies provide further evidence (should it be needed) of the presence and importance of the platelet in thrombosis.

Conclusions and further perspectives

Without prophylaxis against venous thromboembolism, the risk of DVT after surgery to the hip usually exceeds 50%, and that of PE ranges from 4 to 25%. Moreover, the frequency of postoperative PE of perhaps 4% remains a common cause of death[61]. Furthermore, increasing evidence that thrombotic episodes may also precede the diagnosis of cancer, thus representing a potential marker for occult malignancy[62]. These and other findings add weight to the hypothesis that 'high risk' patients should be placed on antiplatelet prophylaxis. In the absence of a reliable marker of platelet activation that is of use to epidemiologists, studies of the platelet in DVT and PE fall back on the pharmacological and laboratory approaches.

Clinical trials have recently demonstrated the effectiveness of aspirin in reducing the risk of DVT and PE. Increased levels of soluble P-selectin at the time of, and post-DVT, are one of the very few instances where the laboratory may have something concrete to offer. Interestingly, increased levels of soluble P-selectin predict the development of arterial thrombosis[63,64]. Whether or not levels of this new platelet marker predict the development of venous thrombosis (whether spontaneous, postpartum or postopertive) remains to be seen. Likewise, the efficacy of GpIIb/IIIa antagonists are now established therapeutics in acute coronary syndromes: time may tell if their effectiveness extends to venous thromboembolic disease.

References

1 Donne A. De l'origine des globules du sang, de leur mode de formation et de leur fin. *Compt Rend Acad Sci* 1842; 3: 366–368.
2 Virchow RLK. *Gesammelte abhandlungen zur wissenschaftlichen medizin.* Frankfurt: Meidinger Sohn; 1856: 219–732.
3 Marra DE, Simoncini T, Liao JK. Inhibition of vascular smooth muscle cell proliferation by sodium salicylate mediated by upregulation of p21–Waf1 and p27–Kip-1. *Circulation* 2000; 201: 2124–2130.
4 Elwood PC, Hughes C, O'Brien JR. Platelets, aspirin, and cardiovascular disease. *Postgrad Med J* 1998; 74: 587–591.
5 Anon. Report of the steering committee of a trial sponsored by the MRC. Effect of aspirin on postoperative venous thrombosis. *Lancet* 1972; 11: 441–445.
6 Morris GK, Mitchell JRA. Preventing venous thromboembolism in elderly patients with hip fractures: studies of low-dose heparin, dipyridamole, aspirin and flurbiprofen. *Br Med J* 1977; 1: 535–537.
7 Harris WH, Salzman EW, Athanasoulis CA, Waltman AC, DeSanctis RW. Aspirin prophylaxis of venous thromboembolism after total hip replacement. *N Engl J Med* 1977; 297:1246–1249.
8 Alfaro MJ, Paramo JA, Roche E. Prophylaxis of thromboembolic disease and platelet related changes following total hip replacement: a comparative study of aspirin and heparin-dihydroergotamine. *Thromb Haemost* 1986; 56: 53–56.
9 Salzman EW, Harris WH, DeSanctis RW. Reduction in venous thromboembolism by agents affecting platelet function. *N Engl J Med* 1971; 284: 1287–1292.

Cambridge Books Online © Cambridge University Press, 2009

10 Antiplatelet triallists' collaboration. Collaborative overview of randomised trials of antiplatelet therapy. III: Reduction in venous thrombosis and pulmonary embolism by antiplatelet prophylaxis among surgical and medical patients. *Br Med J* 1994; 308: 235–246.
11 Cohen AT, Skinner JA, Kakkar VV. Antiplatelet treatment for thromboprophylaxis: a step forward or backwards? *Br Med J* 1994; 309:1213–1215.
12 Collins R, Baigent C, Sandercock P, Peto R et al. Antiplatelet therapy for thromboprophylaxis: the need for careful consideration of the evidence from randomised trials. *Br Med J* 1994; 309: 1215–1217.
13 Pulmonary Embolism Prevention (PEP) Trial Collaborative Group. Prevention of pulmonary embolism and deep vein thrombosis with low dose aspirin. Pulmonary Embolism Prevention (PEP) trial. *Lancet* 2000; 355: 1295–1302.
14 Bernant A, Lale A, Herbert JM. Aurin tricarboxylic acid inhibits experimental venous thrombosis. *Thromb Res* 1994; 74: 617–627.
15 Kakkar VV, Welzel D, Murray WJG, Malone P, Jones D. Possible mechanism of the synergistic effect of heparin and dihydroergotamine. *Am J Surgery* 1985; 150(4A): 33–38.
16 Wu KK, Barnes RW, Hoak JC. Platelet hyperaggregability in idiopathic recurrent deep vein thrombosis. *Circulation* 1976; 53: 687–691.
17 Neiwiarowski S, Thomas DP. Platelet Factor 4 and adenosine diphosphate release during human platelet aggregation. *Nature* 1969; 222: 1269–1270.
18 Moore S, Pepper DS, Cash JD. The isolation and characterisation of platelet-specific B-globulin (β-thromboglobulin) and the detection of antiurokinase and antiplasmin released from thrombin-aggregated washed human platelets. *Biochem Biophys Acta* 1975; 379: 360–369.
19 De Boer AC, Genton E, Turpie AGG. Chemistry, measurement and clinical significance of platelet specific proteins. *CRC Crit Revs Clin Lab Science* 1982; 18: 1183–1211.
20 Chong BH, Murray B, Berndt MC, Dunlop LC. Plasma P-selectin is increased in thrombotic consumptive disorders. *Blood* 1994; 83: 1535–1541.
21 Blann AD, Lip GYH. Hypothesis: is soluble P-selectin a new marker of platelet activation? *Atherosclerosis* 1997; 128: 135–138.
22 Kaser-Glanzman R, Jakabova M, Luscher EF. Isolation and some properties of the heparin neutralising factor (PF4) released from human blood platelets. *Experimentia* 1972; 28:1221–1223.
23 Kaplan KL, Owen J. Plasma levels of beta thromboglobulin and platelet factor four as indices of platelet activation in vivo. *Blood* 1981; 57: 199–202
24 Dawes J, Smith RC, Pepper DC. The release, distribution and clearance of human beta thromboglobulin and Platelet Factor 4. *Thromb Res* 1978; 12: 569–582.
25 Kaplan KL, Broekman J, Chernoff A, Lesznik GR, Drillings M. Platelet alpha granule proteins: studies on release and subcellular localisation. *Blood* 1979; 53: 604–618.
26 Minar E, Ehringer H. Influence of acetylsalicylic acid (1.0g/day) on platelet survival time, beta thromboglobulin and platelet factor 4 in patients with peripheral arterial occlusive disease. *Thromb Res* 1987; 45: 791–802.
27 Zahavi J, Kakkar VV. Beta-thromboglobulin – a specific marker of in-vivo platelet release reaction. *Thromb Haemost* 1980; 44: 23–29.
28 Toth L, Szenasi P, Varsanyi MN et al. Elevated levels of plasma and urine beta thromboglobulin and thromboxane B2 as markers of renal platelet hyperactivation in diabetic neuropathy. *Haemostasis* 1992; 22(6): 334–339.
29 Al-Mondhiry H. Beta-thromboglobulin and platelet factor 4 in patients with cancer. *Am J Haematol* 1983; 14: 105–111.
30 Moore S, Pepper DS. The isolation of a platelet specific β-thromboglobulin and the detection of antiurokinase and antiplasmin released from thrombin aggregated washed human platelets. *Biochem et Biophysica Acta* 1975; 379: 360–369
31 Walz DA. Platelet released proteins as molecular markers for the activation process. *Semin Thromb Haemost* 1984; 10: 270–279.
32 Stenberg PE, Schuman MA, Levine SP, Bainton DF. Redistribution of alpha granules and their contents in thrombi stimulated platelets. *J Cell Biol* 1984: 98; 748–760.
33 Wagner DD. The Weibel–Palade body: the storage granule for von Willebrand factor and P-selectin. *Thromb Haemost* 1993; 70: 105–110.
34 Kawabata K, Nagake Y, Shikata K et al. Soluble P-selectin is released from activated platelets in vivo during haemodialysis. *Nephron* 1998; 78: 148–155.
35 Li N, Wallen NH, Hjemdahl P. Evidence for prothrombotic effects of exercise and limited protection by aspirin. *Circulation* 1999; 100: 1374–1379.
36 Connor CM, Gurbel PA, Serebruany VL. Usefulness of soluble and surface bound P-selectin in detecting platelet activity in patients with congestive heart failure. *Am J Cardiol* 1999; 83: 1345–1349.
37 Jilma B, Blann A, Pernerstorfer T et al. Regulation of adhesion molecules during human endotoxaemia. No acute effects of aspirin. *Am J Resp Crit Care Med* 1999; 159: 857–863.
38 Serebruany VL, Lowry DR. Take an aspirin or have a drink: antecedent aspirin and moderate alcohol consumption affect baseline platelet characteristics in patients with acute myocardial infarction. *Thromb Haemost* 2000; 84: 933–934.
39 Smith RC, Duncanson J, Ruckley CV et al. Beta thromboglobulin and deep vein thrombosis. *Thromb Haemost* 1978; 39: 338–345.
40 Cella G, Zahavi J, de Haas HA, Kakkar VV. Beta thromboglobulin, platelet production time and platelet function in vascular disease. *Br J Haematol* 1979; 43: 127–136.
41 Hirose M, Inada K. Postoperative deep vein thrombosis. Early diagnosis and prophylaxis. *Jap Circulation J* 1984; 48: 111–117.
42 Farrell RJ, Duffy MJ, Duffy GJ. Serum fragment E and plasma beta thromboglobulin in patients with acute pulmonary embolism and non-thromboembolic lung disease. *Thromb Res* 1982; 27: 231–234.

Cambridge Books Online © Cambridge University Press, 2009

43 Van Hulsteinjn H, Briet E, Koch C, Hermans J, Bertina R. Diagnostic value of fibrinopeptide A and beta thromboglobulin in acute deep vein thrombosis and pulmonary embolism. *Acta Med Scand* 1982; 211: 323–330.
44 Sakaguchi S, Furuta Y, Kameda T. Haemopathological changes in recurrent deep vein thrombosis of the extremities. *J Cardiovasc Surg* 1982; 23: 117–122.
45 Wojciechowski J, Olausson M, Korsan-Bengtsen K. Fibrinopeptide A, beta thromboglobulin, and fibrin degradation products as screening test for the diagnosis of deep vein thrombosis. *Haemostasis* 1983; 13: 254–261.
46 Lane DA. Ireland H, Wolff S, Grant R, Jennings S, Allen-Mersh T. Plasma concentrations of fibrinopepetide A., fibrinogen fragment Bbeta1–42 and beta thromboglobulin following total hip replacement. *Thromb Res* 1982; 26: 111–118.
47 Paramo JA, Rocha E. Deep vein thrombosis and related platelet changes after total hip replacement. *Haemostasis* 1985; 15: 389–394.
48 Owen J, Kvam D, Nossel HL, Kaplan KL, Kernoff PBA. Thrombin and plasmin activity and platelet activation in the development of venous thrombosis. *Blood* 1983; 61: 476–482.
49 Bolton AE, Cooke ED, Lekhwani CP, Bowcock SA. Urinary beta thromboglobulin levels as a diagnostic marker for post-operative deep vein thrombosis. *Thromb Res* 1980; 19: 249–255.
50 Van Hulsteinjn, Bertina R, Briet E. A one-year follow up of plasma fibrinopeptide A and beta thromboglobulin after deep vein thrombosis and pulmonary embolism. *Thromb Res* 1982; 27: 225–229
51 Smith A, Quarmby JW, Collins M, Lockhart SM, Burnand KG. Changes in levels of soluble adhesion molecules and coagulation factors in patients with deep vein thrombosis. *Thromb Haemost* 1999; 82: 1593–1599.
52 Blann AD, Noteboom WMP, Rosendaal FR. Increased soluble P selectin levels following deep venous thrombosis: cause or effect? *Br J Haematol* 2000; 108: 191–193.
53 Douglas JT, Blamey SL, Lowe GDO, Carter DC, Forbes CD. Plasma beta-thromboglobulin, fibrinopeptide A and B beta 15–42 antigen in relation to post operative DVT, malignancy and stanazolol treatment. *Thromb Haemost* 1985; 53: 235–238.
54 Rabinov K, Paulin S. Roentgen diagnosis of venous thrombosis in the leg. *Arch Surg* 1972; 22: 490–494.
55 Sundrehagen E, Urdal P, Heggli DE, Lindegaard MW, Jacobsen E. Radiolabelling of platelets with technetium-99m. *Thromb Res* 1990; 57: 737–745.
56 Morimoto Y, Sugimoto T, Okada M, Mukai T. Clinical assessment of vascular thrombosis using indium-111 platelet scintigraphy. *Angiology* 2000; 51: 61–68.
57 Muto P, Lastoria S, Varella P et al. Detecting deep venous thrombosis with technetium-99m-labelled synthetic peptide P280. *J Nucl Med* 1995; 36: 1384–1391.
58 Taillefer R, Edell S, Innes G, Lister-James J. Acute thromboscintigraphy with (99m) Tc apcitide: results of the phase 3 multicentre clinical trial comparing 99mTc apcitide scintigraphy with contrast venography for imaging acute DVT. *J Nucl Med* 2000; 41: 1214–1223.
59 Mousa SA, Bozarth JM, Edwards S, Carroll T, Barrett J. Novel technetium-99m-labelled platelet gpIIb/IIIa receptor antagonists as potential imaging agents for venous and arterial thrombosis. *Coron Artery Dis* 1998; 9:131–141.
60 Knight LC, Romano JE, Maurer AH. In vitro platelet binding compared with in vivo thrombus imaging using alpha (IIb) beta 3 targeted radioligands. *Thromb Haemost* 1998; 80: 845–851.
61 Sors H, Meyer G. Place of aspirin in prophylaxis of venous thromboembolism. *Lancet* 2000; 355: 1288–1289.
62 Donati MB. Cancer and thrombosis. *Haemostasis* 1994; 24: 128–131.
63 Blann AD, Faragher EB, McCollum CN. Increased soluble P-selectin in ischaemic heart disease: a new marker for the progression of atherosclerosis. *Blood Coagul Fibrinol* 1997; 8: 383–390.
64 Ridker PM, Buring JE, Rifai N. Soluble P-selectin and the risk of future cardiovascular events. *Circulation* 2001; 103: 491–495.

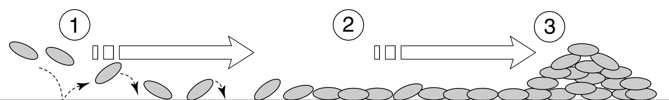
Cambridge Books Online © Cambridge University Press, 2009

50

Gene regulation of platelet function

Thomas J. Kunicki

The Scripps Research Institute, La Jolla, California, USA



Stage 1. Tethering
GP Ib complex and von Willebrand factor
Stage 2. Adhesion
Integrin $\alpha_2\beta_1$ and GP VI
Stage 3. Cohesion
Integrin $a_{IIb}\beta_3$ and fibrinogen or von Willebrand factor

Fig. 50.1. Platelet thrombus formation. The process of platelet thrombus formation can arbitrarily be divided into three stages: (i) Transient adhesion (or tethering) mediated by binding of the platelet glycoprotein (GP) Ib complex to Von Willebrand Factor (VWF) adsorbed to the matrix; (ii) adhesion with synchronous platelet activation, mediated in the case of collagens by the integrin $\alpha_2\beta_1$ and platelet GPVI; and (iii) the formation of a platelet aggregate (or cohesion) mediated by the binding of either or both fibrinogen and VWF to the activated integrin $\alpha_{IIb}\beta_3$.

The process of hemostasis or thrombus formation mediated by blood platelets is complex and can involve several alternative receptor–ligand interactions. To facilitate an analysis of the mechanisms that control platelet function, we can arbitrarily divide the process of platelet hemostasis into three stages (Fig. 50.1).

The first stage is a transient tethering of platelets to the damaged subendothelium. This initial transient platelet adhesion requires the specific binding of the plasma protein Von Willebrand factor (VWF) to the membrane receptor glycoprotein Ib–IX–V complex (GP Ib complex). This association is critical to normal platelet function in preventing blood loss and is a requisite step in the adhesion of platelets to exposed thrombogenic materials in regions of atherosclerotic plaque rupture, which sets the stage for thrombus formation.

In the second stage, platelets form a more stable monolayer over the thrombogenic surface that can be mediated by a variety of receptors, depending on the nature of the extracellular matrix and the adhesive proteins that are present. In damaged areas of the blood vessel wall, where there is an abundance of collagens, this stage is frequently mediated by the interaction of collagen receptors, such as the integrin $\alpha_2\beta_1$ and the platelet-specific receptor GPVI. Integrin $\alpha_2\beta_1$ primarily supports platelet attachment to collagen, while GPVI and to a lesser extent $\alpha_2\beta_1$ work in concert to transduce signals through the membrane. The engagement of these receptors culminates in the activation of platelets and signals the onset of the next stage. This is also the stage at which the activated platelet surface catalyses the formation of thrombin serving as a nidus for the formation of a fibrin clot.

Platelet cohesion follows in a third stage resulting in the generation of platelet-rich thrombi mediated by the binding of fibrinogen or vWF to the activated platelet integrin $\alpha_{IIb}\beta_3$.

To fully appreciate the impact of genetic dimorphisms on platelet function *vis-à-vis* each of these stages, it is important to compare and contrast the regulatory properties of the several receptor genes that are restricted to, or differentially regulated within, the megakaryocyte lineage.

Gene regulation in the megakaryocyte lineage

Compared to the other hematopoietic lineages, gene expression in megakaryocytes is difficult to study, because these cells are less common, more fragile and quickly differentiate to a committed apoptotic fate. Consequently, the regulation of megakaryocyte-specific genes has been investigated predominantly in pluripotent human hematopoietic cell lines that have megakaryocyte features and express several platelet-specific genes, e.g. GPIbα, GPIbβ,

GPIX, α_{IIb}, PF-4, GPVI or GPV. The cell lines most commonly used for this purpose are erythro-megakaryocytic and include K562, HEL, Dami and CHRF-288–11.

The recent cloning of the megakaryocyte inducer thrombopoietin may now enable the development and expansion *ex vivo* of primary megakaryocytes in quantities sufficient to perform biochemical analyses and to characterize megakaryocyte developmental regulators. This will facilitate the characterization of megakaryocyte-specific transcriptional regulators, where they exist. In the interim, our current understanding is derived from the study of the aforementioned pluripotent, hematopoietic cell lines.

Megakaryocytic-specific gene promoters, including α_{IIb}, PF-4, GPVI, GPV and GPIbα, have a number of common characteristics. The core promoters are generally short (500 base pairs), lack CCAAT or TATA consensus sequences, and have functional GATA, Sp-1 and Ets *cis*-acting sites. GATA and Ets factors are not exclusive to the megakaryocyte lineage but are also critical for gene expression in erythroid cells and are important for the regulation of genes like PECAM-1and P-selectin, which are expressed in both megakaryocytes and endothelial cells.

GATA

The GATA family of transcription factors has in common a high degree of sequence homology within zinc finger DNA binding domains and specific binding to the motif (5′-GATAA/G-3′). GATA-1 is primarily expressed in erythroid, megakaryocytic, mast, and eosinophilic cells[1–4]; GATA-2 is expressed in megakaryocytes, endothelial cells and other cell types[3,5,6]; and GATA-3 is expressed in T lymphocytes, erythrocytes, and brain (during development)[7–8]. GATA-1 is only expressed early in megakaryocyte maturation while GATA-2 expression persists, and maturation-dependent differences in the utilization of GATA-1 and GATA-2 could play an important role in protein expression[9].

GATA-1 directs the differentiation of hematopoietic progenitors along either the erythroid or the megakaryocytic pathway. For example, in the primitive myeloid cell line 416B, overexpression of GATA-1 leads to megakaryocytic differentiation[10]. GATA-2 and GATA-3 transcripts in these cells remain undetectable, suggesting that GATA-2 and GATA-3 lie upstream of GATA-1. Enforced expression of GATA-2 or GATA-3 in the same cells will then also induce megakaryocytic differentiation, presumably via induction of GATA-1, a finding consistent with the conclusion that GATA-1 is a dominant regulator of megakaryocyte maturation.

Initial murine knock-out experiments did not show a direct link between GATA-1 and megakaryocytic differentiation[11,12]. GATA-1 (−/−) mice exhibited severe anemia and total arrest of erythroid differentiation at the proerythroblastic stage[13,14]. These erythroid precursors later died by apoptosis. More recently, megakaryocyte-specific GATA-1 knock-out mice have provided a more informative phenotype. These mice have decreased peripheral blood platelet counts, deregulated proliferation of megakaryocytes and a block in their terminal cytoplasmic maturation[15]. These mice, however, express normal amounts of α_{IIb} and MPL (the thrombopoietin receptor) and 50-fold higher levels of GATA-2, suggesting that GATA-2 compensates for the loss of GATA-1 and that GATA-1 negatively regulates GATA-2 during normal erythroid maturation[16].

Ets

The Ets factors are a family of transcription factors that regulate expression of many hematopoietic genes. The prototype, Ets-1, was given its name because it is expressed as part of an oncogenic protein by the E-26 virus[17]. Ets factors have in common an 82 amino acid motif (the Ets domain) that binds to DNA sequences containing a consensus 5′-GGAA/T-3′[18].

One member of the Ets family, Friend's leukemia integration-1 (Fli-1; ErgB) is over expressed in erythroleukemia cells of mice infected with Friend's leukemia virus. Recent experiments suggest that Fli-1 might play a role in the regulation of megakaryocytic genes. The human erythro-megakaryocytic cell line K562, which normally lacks Fli-1, shows an increased expression of megakaryocytic features when transfected with a vector expressing Fli-1[19]. Moreover, the α_{IIb} promoter can be transactivated by Fli-1in HeLa cells transiently cotransfected with α_{IIb} promoter constructs[19].

The Ets factor PU.1 has been identified in mature megakaryocytes, and binding sites for this factor are present in megakaryocytic genes, such as α_{IIb} and β-thromboglobulin. Overexpression of PU.1 in mice induces erythroleukemia through blockage of erythroid differentiation[20], and the overexpression of PU.1 on these murine erythroleukemia cells reduces their NF-E2 expression and the DNA-binding activity of GATA-1[21]. PU.1 can also transactivate the promoter for the megakaryocyte-specific platelet basic protein.

Cambridge Books Online © Cambridge University Press, 2009

Fig. 50.2. The platelet GPIb complex. Four distinct gene products (GPIbα, GPIbβ, GPIX and GPV) assemble to form a functional heptamer known as the GPIb complex. Each of these polypeptides contains a transmembrane region and cytoplasmic domain. The functional keystone of the complex is GPIb, composed of the larger GPIbα (containing the VWF binding site) that is disulfide bonded to the smaller GPIbβ. Each complex is composed of: two GPIb, two GPIX and one GPV subunit. Each of the subunit genes has in common the presence of a variable number of leucine-rich motifs.

Stage 1. Transient platelet tethering mediated by the GP Ib complex

The GPIb complex (Fig. 50.2) is a heptamer composed of four distinct gene products: two molecules of GPIbα, two of GPIbβ, two of GPIX and one of GPV. VWF is directly bound by the GPIbα subunits, each of which is disulfide-linked to a GPIbβ subunit. GPIX is noncovalently associated, and GPV is loosely associated with the complex. The GPIbα, GPIX, and GPV genes are restricted in their expression to the platelet lineage and appear late in megakaryocytopoiesis.

The Bernard–Soulier syndrome is a human bleeding disorder associated with absent or significantly reduced GPIb complex on the platelet surface. Numerous genetic causes of BSS have been characterized at a molecular level, including fourteen due to mutations in GPIbα, five resulting from defects in GPIX, and four ascribed to abnormalities in GPIbβ[22–27]. There are no reports of BSS resulting from genetic differences in the GPV gene. From reconstitution studies, we now know that GPIbα, GPIbβ and GPIX are all necessary for the expression of the complex on the cell surface. GPV is not absolutely required but does promote complex stability, and the absence of GPV in transgenic mice results in an increase in GPIb complex activity. New insight in the function of GPV have been derived from murine knockout experiments, whereby it has been shown that GPV actually participates in the control of the binding activity of the GPIb complex (see below).

A common feature of the GPIb complex genes is a variable number of leucine-rich repeat motifs (LRM) in all four subunits. GPIbα (135 kd; 610 amino acids) has seven, GPIbβ (25 kd; 181 amino acids) and GPIX (22 kd; 160 amino acids) each have a single LRM, and GPV (82 kd; 544 amino acids) has 15. It is believed that the LRM play an important role in protein–protein interactions. Based on sequence comparisons, it appears that GPIX is the ancestral progenitor of the other members of this LRM family.

Comparative studies of transcriptional regulation of the four subunits has contributed to our understanding of subunit inter-relationships and the identification of those subunits which are rate limiting during complex assembly. Like the polypeptides, the genes encoding each subunit have several characteristics in common. The coding region of each gene, except GPIbβ, is contained within a single exon. In the case of GPIbβ, a small intron interrupts the signal sequence coding region[28]. In each gene, as small intron is located 5′ to the translation start site. Because of the presence of only one or two introns, the genes are relatively small. For example, the largest, GPIbα, spans only 4 kb, including the 5′ and 3′ regulatory regions[29,30]. Though they are structurally similar, however, the genes are dispersed throughout the human genome: GPIbα is located on chromosome 17 (17p12)[28]; GPIbβ is on chromosome 22 (22q11.2)[28,31]; and the genes for GPIX and GPV are located on different regions of chromosome 3 (3q29 and 3q24, respectively[32]).

GPIbα

GPIbα, the largest protein of the complex, contains, at the amino-terminus of its extracellular domain, the binding site for VWF[33] and α-thrombin[34] and has binding sites for 14.3.3ζ[35] and the actin cytoskeleton[36] in its short cytoplasmic region.

Transcription of the GPIbα gene, as well as the other members of the complex in megakaryocytes is controlled by factors of the GATA and Ets families[15], and FOG-1 (Friend of GATA-1) is a necessary cofactor controlling the lineage specificity of GPIbβ gene expression[37]. The importance of the GATA-1 site(s) is reflected by the fact that

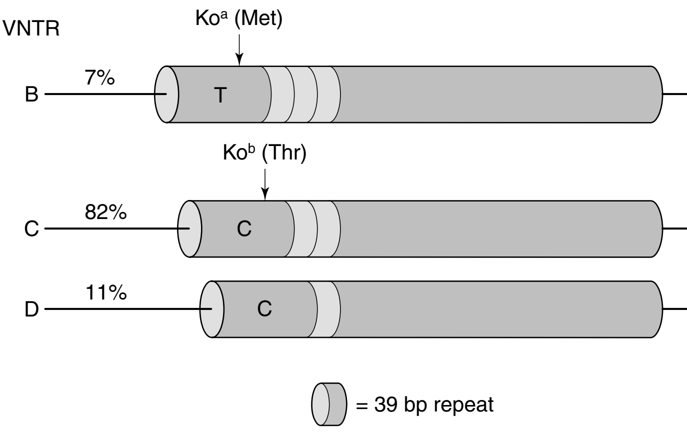
Cambridge Books Online © Cambridge University Press, 2009

mutations of the GATA-1 site in both the GPIbα and GPIX genes *in vitro* essentially eliminates transcription controlled by these promoters[9,38], and a point mutation of the GPIbβ GATA-1 site has been shown to be a cause of one case of BSS[39]. Moreover, recent murine knockout experiments have shown that mice lacking GATA-1 express platelets with a BSS phenotype[15].

At least three polymorphisms influence the function, expression and immunogenicity of the GPIb complex genes, particularly the GPIbα subunit. A variable number of tandem repeats (VNTR) polymorphism within the mucin-like macroglycopeptide region of GPIbα (Fig. 50.3, top panel) results in the duplication of a 13-amino acid sequence either once (VNTR D), twice (VNTR C), thrice (VNTR B) or four times (VNTR A), and results in a polypeptide length of 610, 623, 636 or 649 amino acids, respectively[40,41]. Because these repeats are rich in proline, serine and threonine, they can be potentially glycosylated, and each repeat could add up to 32 angstroms to the length of the GPIbα extracellular domain[40]. In a simple model, this could extend the GPIbα binding sites for VWF and thrombin further above the plane of the plasma membrane, increasing the avidity for these ligands and accounting for the observed increased risk for acute coronary artery disease associated with the longer variants, VNTR A and VNTR B[42,43].

A second clinically relevant GPIbα polymorphism is the threonine to methionine substitution at amino acid 145, within the region of the ligand-binding LRM[44,45]. This dimorphism is the basis of the HPA-2 platelet alloantigen system (Fig. 50.4), and the allele frequencies for the Thr and Met alleles in a typical Caucasian population are 90% and 10%, respectively[44,45].

A third polymorphic variation in the region surrounding the translation start site, at position −5 from the initiator ATG codon (where either T or C is present) (Fig. 50.3, bottom panel), was shown to influence the levels of the receptor expressed on the surfaces of transfected cells and platelets[46,47]. The −5C allele has a gene frequency of about 0.15 in several human ethnic populations and may be a determinant of platelet responsiveness because it influences GPIbα expression. The presence of the −5C allele increases the mean level of GPIbα on the platelet plasma membrane (roughly, a 50% increase in homozygous individuals; and a 33% increase in heterozygous individuals) presumably because it enhances the interaction of the cellular protein translation machinery with the adjacent Kozak sequence[46].



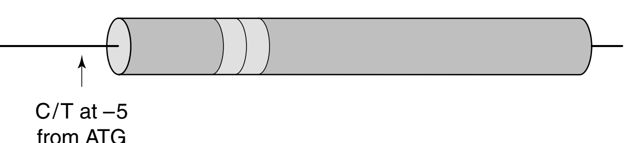


Fig. 50.3. Polymorphisms of the GPIbα subunit. Three polymorphisms are relevant to the expression and function of GPIbα. (Top panel) The variable number of tandem repeats (VNTR) polymorphism is characterized by the expression of four (VNTR A; not shown), three (VNTR B), two (VNTR C) or one (VNTR D) copy of a 39-base pair repeat sequence. In a typical Caucasian population, the gene frequencies of each allele are: A (<0.01); B (0.07); C (0.82); and D (0.11). Another dimorphism, the Met/Thr at residue 145 (nucleotide T/C substitution), gives rise to the Ko^a and Ko^b alloantigens and is linkage disequilibrium with the VNTR polymorphism. Thus, the VNTR A and B alleles express only Met-145, while the VNTR C and D alleles express only Thr-145. (Bottom panel) In GPIbα, a C/T substitution at a position five nucleotides upstream from the ATG start codon influences an adjacent Kozak sequence and thus the rate of translation of the mRNA transcript by the cell machinery. On average, the relationship of GPIb levels to genotype at this position is: T/T = 1.0; T/C = 1.3; C/C = 1.5.

GPIbβ

The functional role of GPIbβ in the complex has not been clearly defined, although in vitro evidence suggests that this protein can act as a complex-specific chaperone that protects GPIbα from lysosomal degradation while it is transported to the membrane[48].

Moran et al.[26] described recently a patient with a novel, homozygous, single-nucleotide substitution (Glycine-

Cambridge Books Online © Cambridge University Press, 2009

Glycoprotein Ib alleles

GP Ib α Residue 145	VNTR	f	HPA-	a.k.a
Thr	C	0.82	2a	Ko[b]
Thr	D	0.11	2a	Ko[b]
Met	B	0.07	**2b**	**Ko[a], Sib[a]**
Met	C	<0.01	**2b**	**Ko[a], Sib[a]**
Met	A	<0.01	**2b**	**Ko[a], Sib[a]**
GP Ib β Residue 15		**f**	**HPA-**	**a.k.a**
Gly		>0.99	11aw	
Glu		<0.01	**11bw**	**ly[a] (+)**

Fig. 50.4. Glycoprotein Ib alleles. For GPIbα, five alleles can be defined based on the threonine/methionine dimorphism at residue 145 and the VNTR. For GPIbβ, two alleles are distinguishable by the glycine/glutamate substitution at residue 15. Abbreviations: a.k.a, also known as; f, gene frequency; HPA, human platelet antigen; and VNTR, variable number of tandem repeats. Gene frequencies for a typical Caucasian population are indicated.

159→alanine) in the coding region for GPIbβ at a tryptophan codon (TGG), which results in the premature termination of translation at amino acid 21. No sequence abnormalities were observed in either the GPIbα or the GPIX subunit of the complex. Although GPIbα, GPIX, and GPV were undetectable on the platelet surface, GPIbα was readily demonstrated in platelets. Moreover, soluble GPIbα was present in plasma. The defective GPIbβ is thus unable to support the expression of a functional complex at the platelet surface. Thus, GPIbβ affects the surface expression of the GPIb–IX complex by failing to support the insertion of GPIbα and GPIX into the platelet membrane[26].

This critical role for GPIbβ as a chaperone was confirmed by Strassel and coworkers[27] who identified a different single amino acid substitution in GPIbβ (Asparagine-63→Threonine) that also results in the inhibition of expression of cotransfected wild-type GPIbα in CHO cells.

A rare Gly→Glu substitution at residue 15 gives rise to the HPA-11bw alloantigen (ly[a])[49].

GPIX.

Like GPIbβ, GPIX is thought to act as a complex-specific chaperone that protects GPIbα from lysosomal degradation while it is transported to the membrane[48].

Bastian et al.[9] identified an Ets site (ACTTCCT) located between −35 and −49 relative to the GPIX transcriptional start site that, when disrupted, reduced promoter activity in transiently transfected HEL cells. The identity of the protein(s) that binds to this GPIX Ets site remains to be determined, however, *in vitro* binding studies had shown that Ets-1 and Ets-2 can bind to the α_{IIb} promoter sites, and there is some indication that one or both of these factors regulate GPIX promoter activity.

The oncogenic protein Fli-1 can transactivate the GPIX promoter when an intact GPIX Ets site is present. Comparative studies showed that Fli-1 was also able to transactivate the GPIbα and, to a lesser extent, the α_{IIb} promoter. Although Fli-1 protein has been identified in platelet lysates, it seems unlikely that Fli-1 is translated de novo in platelets, but more probable that the presence of Fli-1 in platelets represents residual protein synthesized in the megakaryocyte.

Negative regulation is emerging as a common theme in megakaryocyte-specific promoters. Transcriptional silencer domains have been identified upstream of the human PF-4 gene and within the rat PF-4 and human αIIb promoters. The GPIX promoter has a weak negative regulatory domain between −686 and −423 and a strong negative domain between −311 and −203. A comparison of negative regulatory domains in the megakaryocyte gene promoters led to the identification of a handful of potential regulatory motifs. Prandini *et al.*[50] identified transcriptional repression sites in the α_{IIb} promoter: 5′-TGAGT-3′ at −120 to −116 and 5′-CCCTTTGCTC-3′ −102 to −93, relative to the transcription start site (see below). An exact duplicate of the α_{IIb} 5′-TGAGT-3′ hexamer is found in the weak regulatory domain of the GPIX promoter at −455 to −451. In addition, within the strong GPIX negative repressor domain, there are two sequences (−282 to −273 and −263 to −254) that are somewhat similar (3/9 mismatches) to the α_{IIb} CCTTTGCTC sequence.

GPV

GPV is thought to play a contributory role in the high-affinity binding of thrombin to the platelet[51] but has never been considered essential for the surface expression of a functional complex[52]. The GPV gene is comprised of a short intron in the 5′ untranslated region and a coding sequence contained entirely in the second exon. The 5′-flanking region contains consensus binding sites for GATA, Ets transcription factors and Sp1. The rat and mouse genes are identical in structure and have conserved many of the putative human regulatory sequences. GPV appears late in megakaryocyte differentiation and is absent from most megakaryocytic leukemic cell lines, unlike the other platelet-specific proteins described above. HEL or K562

Cambridge Books Online © Cambridge University Press, 2009

cells are probably blocked at early stages of differentiation, because they express both erythroid and megakaryocytic markers, so that neither of these cell lines expresses GPV, even at the mRNA level. Dami cells, on the other hand, despite recent confirmation that they are subclones of HEL cells, do express GPV, accompanied by increased levels of GPIb-IX (relative to HEL cells). This implies that GPV functions in later stages of megakaryocyte maturation and would necessarily respond to late stage growth or transcription factors.

A repressor domain (-903 to -816) contains a sequence homologous to the silencer region of the α_{IIb} promoter. α_{IIb} -specific expression relies on the activity of this strong repressor, and mutation of this element leads to a ubiquitous activity of the promoter. On the other hand, deletion of this repressor region in the GPV gene does not reverse cell specificity[53]. The core promoter ($-103/+1$) contains two Ets binding sites, Ets-66 and Ets-42, and a GATA site, GATA-71. The GATA/Ets tandem of the core promoter resembles those of the other megakaryocyte-specific genes, α_{IIb}, PF4, GPIbα, β-TG, GPIX, and MPL. Mutation analysis of the GATA/Ets tandem showed that this tandem is essential for GPV transcription and that both sites are important for full transcriptional activity of the GPV promoter in the megakaryocytic lineage. The most proximal Ets site (Ets-42) is most critical for maintaining basal activity, and deletion of this site abolished transcriptional activity.

Signal transduction through the GPIb complex

GPIbα is associated through its cytoplasmic domain with actin-binding protein (ABP; filamin)[54,55] and the protein 14–3–3ζ[56].

Upon engagement of VWF, the signalling events mediated by the GPIb complex include elevation of cytoplasmic Ca^{2+} and activation of protein kinase C and tyrosine kinases[57–60]. An unidentified tyrosine kinase becomes associated with the GPIb complex as a result of VWF binding to GPIbα[61]. Two tyrosine kinase substrates (76 kd and 32 kd) become phosphorylated[57], the larger perhaps identical to syk, the smaller unidentified[58]. In addition, pleckstrin and myosin light chain are also rapidly phosphorylated by Ser/Thr protein kinases[57], both src and activated phophatidylinositol 3-kinase become associated with the cytoskeleton[60], phosphatidylinositol 4,5-biphosphate is metabolized, phosphatidic acid is synthesized, phospholipase A_2 is activated, and arachidonic acid and thromboxane A_2 (TXA_2) are synthesized[57].

Although none of the cytoplasmic sequences of the GPIb complex contain motifs known to interact with signalling proteins, these regions do interact with the cytoskeleton, and signal complexes may assemble at the junction of the GPIb complex with this scaffold. Signal transduction may be mediated, in part, by the binding of 14–3–3ζ[35]. 14–3–3ζ has been shown to bind to proteins with a conserved RSXSXP motif in which the second serine is phosphorylated[62]. It remains to be determined to what extent 14–3–3ζ is involved in the mediation of signals through the GPIb complex.

Another candidate signalling pathway would require the FcRγ chain[63] and involves Syk. Tandem Src homology 2 (SH2) domains of Syk bind to proteins containing phosphorylated ITAMs, which are not found on any of the four subunits of the GPIb complex. However, platelets do express two ITAM-containing proteins, FcγRIIA, and the FcRγ chain. It is possible that the GPIb complex can bind with either or both of these proteins, allowing the complex to couple to Syk. The contribution of FcRγ chain to signal transduction in platelets is even more extensive, since it has recently been shown to be complexed constitutively to the platelet collagen receptor GPVI (see below). From murine knockout experiments, we now know that both the FcRγ chain and Syk are essential for platelet activation by collagen. Importantly, in the absence of FcRγ chain, collagen is unable to induce Syk activation, showing the FcRγ chain is a critical upstream regulator of Syk.

These findings lead to a working model[63] in which the GPIb complex associates with FcRγ chain, either constitutively or upon receptor activation, and the Src family kinases Fyn and Lyn phosphorylate FcRγ chain resulting in the binding of Syk through its tandem SH2 domains, activation of Syk, and phosphorylation-dependent activation of PLCγ2. It remains to be proven that PLCγ2 becomes activated downstream of GPIb and, if so, whether tyrosine phosphorylation of PLCγ2 is the sole mechanism by which PLCγ2 may become activated downstream of this receptor.

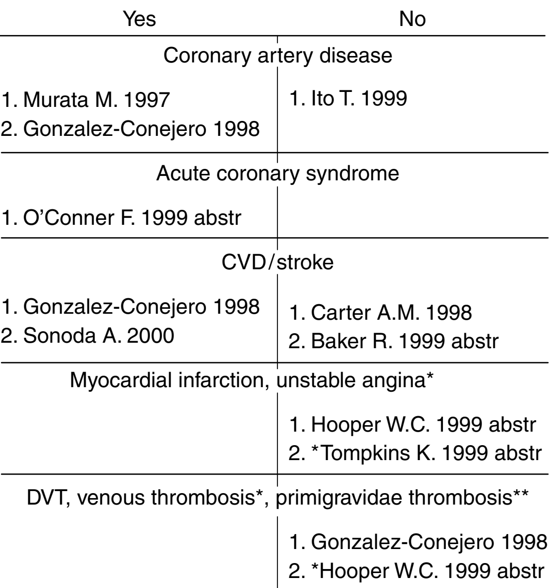
Clinical relevance of GPIb complex polymorphisms

As noted above, there is ample evidence that a high degree of interdependence among the members of the GPIb complex is needed for receptor expression and efficacy. However, the bulk of clinical correlations accumulated to date involve the GPIbα subunit.

Since the GPIb complex is pivotal to the initial tethering of platelets to the extracellular matrix, it is not surprising that structural differences in the keystone subunit, GPIbα, would influence thrombosis in vivo. As summarized in Fig. 50.5, there is substantial evidence for an association between the VNTR B allele and risk of arterial thrombosis and acute adverse outcomes in coronary artery disease. On

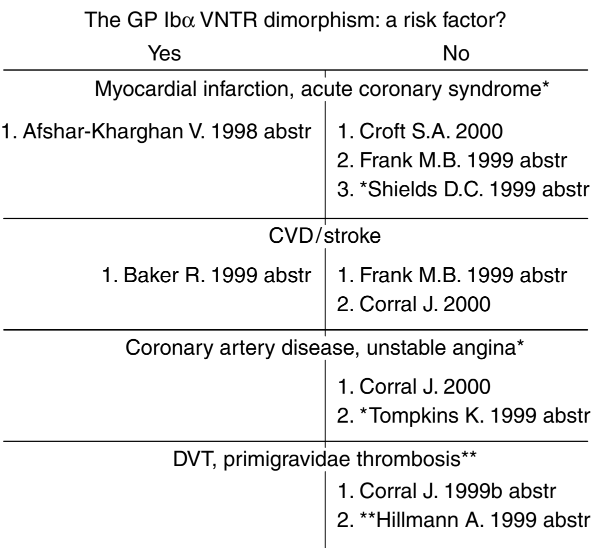
Cambridge Books Online © Cambridge University Press, 2009

The GP Ibα VNTR polymorphism: a risk factor?



Yes	No
Coronary artery disease	
1. Murata M. 1997 2. Gonzalez-Conejero 1998	1. Ito T. 1999
Acute coronary syndrome	
1. O'Conner F. 1999 abstr	
CVD/stroke	
1. Gonzalez-Conejero 1998 2. Sonoda A. 2000	1. Carter A.M. 1998 2. Baker R. 1999 abstr
Myocardial infarction, unstable angina*	
	1. Hooper W.C. 1999 abstr 2. *Tompkins K. 1999 abstr
DVT, venous thrombosis*, primigravidae thrombosis**	
	1. Gonzalez-Conejero 1998 2. *Hooper W.C. 1999 abstr 3. **Hillmann A. 1999 abstr

Fig. 50.5. Correlation between inheritance of the GPIbα VNTR B polymorphism (methionine-145) and risk for adverse outcomes in various thrombotic disorders. Individual studies that confirm (YES) or refute (NO) a correlation between inheritance of the VNTR B allele (presence of methionine-145) and risk for thrombotic disease are indicated. The clinical syndromes include: acute coronary syndrome, acute myocardial infarction, cerebral vascular disease (CVD) / stroke, coronary artery disease, deep vein thrombosis (DVT), primigravidae thrombosis, unstable angina, and venous thrombosis. Data are tabulated as of November 2000.


The GP Ibα VNTR dimorphism: a risk factor?

Yes	No
Myocardial infarction, acute coronary syndrome*	
1. Afshar-Kharghan V. 1998 abstr	1. Croft S.A. 2000 2. Frank M.B. 1999 abstr 3. *Shields D.C. 1999 abstr
CVD/stroke	
1. Baker R. 1999 abstr	1. Frank M.B. 1999 abstr 2. Corral J. 2000
Coronary artery disease, unstable angina*	
	1. Corral J. 2000 2. *Tompkins K. 1999 abstr
DVT, primigravidae thrombosis**	
	1. Corral J. 1999b abstr 2. **Hillmann A. 1999 abstr

Fig. 50.6. Correlation between inheritance of the GPIbα Kozak C dimorphism and risk for adverse outcomes in various thrombotic disorders. Individual studies that confirm (YES) or refute (NO) a correlation between inheritance of the Kozak C allele and risk for thrombotic disease are indicated. The clinical syndromes include: acute coronary syndrome, acute myocardial infarction, cerebral vascular disease (CVD) / stroke, coronary artery disease, deep vein thrombosis (DVT), primigravidae thrombosis and unstable angina. Data are tabulated as of November 2000.

the other hand, risk for venous thrombosis is consistently negative. Aside from two preliminary reports, the majority of studies have failed to find a correlation between the inheritance of the Kozak C genotype (high receptor density) and risk for coronary artery disease (Fig. 50.6). However, a very recent report does provide evidence that there may well be an association with the *severity* of negative outcomes following acute myocardial infarction in younger individuals (≤62 years old)[64].

Stage 2. Stable adhesion to collagens mediated by integrin $\alpha_2\beta_1$ and GPVI

Collagen receptors

Collagens will induce platelet shape change, secretion, and aggregation, accompanied by phosphoinositide hydrolysis, TXA_2 synthesis, phosphorylation of specific protein substrates, and an increase in cytoplasmic Ca^{2+}.

The human α_2 gene

The integrin α_2 subunit is a single chain transmembrane polypeptide that pairs exclusively with the β_1 subunit (Fig. 50.7, left panel). In human, a single copy of the α_2 gene is present in the haploid genome, located on chromosome 5 (5p11–12)[65]. The cDNA sequence published by Takada and Hemler[66] was nearly complete, lacking the bulk of the final exon 30 and a polyadenylation site.

Platelet $\alpha_2\beta_1$ levels among randomly selected individuals can vary up to 10-fold and correlate with differences in adhesiveness to type-I or type-III collagens. We identified linked, allelic polymorphisms within the coding sequence of the α_2 gene that correlate with platelet $\alpha_2\beta_1$ density[67,68]. These observations led to further characterization of the integrin α_2 alleles, summarized schematically in Fig. 50.7 (centre panel). Four α_2 alleles can be defined: Allele 1 (807T/1648G/2531C) is associated with increased levels of $\alpha_2\beta_1$, while Allele 2 (807C/1648G/2531C) and Allele 3

Cambridge Books Online © Cambridge University Press, 2009

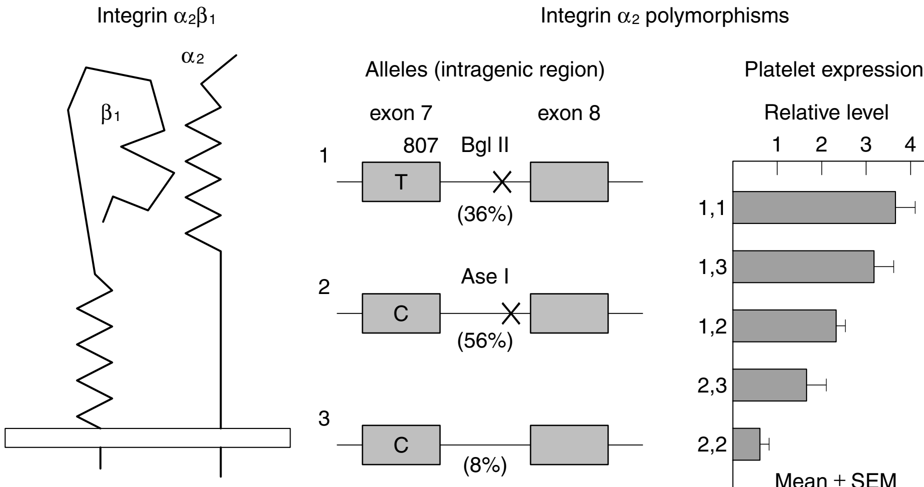


Fig. 50.7. The integrin $\alpha_2\beta_1$. (Left panel) This receptor is a heterodimer composed of non-covalently associated α_2 and β_1 subunits, both of which contain transmembrane regions and cytoplasmic domains. (Centre panel) Three common α_2 alleles can be distinguished by the silent 807 C/T dimorphism within exon 7 and the presence of unique *Bgl II* or *Ase I* restriction sites within the intron separating exons 7 and 8. The gene frequencies of each of these alleles in a typical Caucasian population are: allele 1 = 0.36; allele 2 = 0.56; and allele 3 = 0.08. (Right panel). The relative level of platelet $\alpha_2\beta_1$ is controlled by the inheritance of these three α_2 alleles. For example, individuals who are homozygous for α_2 allele 1 express, on average, fourfold the amount of this receptor that would be found on platelets of individuals who are homozygous for α_2 allele 2.

(807C/1648A/2531C) are each associated with decreased levels of this receptor. The gene frequencies of these three alleles in a typical Caucasian population are: Allele 1, 0.36; Allele 2, 0.56; and Allele 3, 0.08. Two intronic sequence differences (between exons 7 and 8) create unique *Bgl II* and *Ase I* restriction sites[69] that can be used to distinguish each allele by RFLP assay (Fig. 50.7, centre panel). The rate of platelet attachment to type I collagen in whole blood under conditions of high shear rate (1,500/s) is proportional to the density of $\alpha_2\beta_1$ receptors on the platelet surface, as determined by differential inheritance of alleles 1 or (2 + 3) (Fig. 50.7, right panel). These initial findings suggested that α_2 alleles could influence risk of thrombosis or bleeding in relevant disease states.

The very rare allele 4 (gene frequency <0.01) uniquely expresses 2531T, which defines the Sit^a alloantigen (Fig. 50.8). Within the coding sequence, two single point missense dimorphisms have been described: $A_{1648}G$ ($Lys_{505}Glu$) and $C_{2531}T$ ($Thr_{799}Met$) are responsible for the formation of the Br^a/Br^b (HPA-5) and Sit^a alloantigens (HPA-12bw), respectively[70,71] (Fig. 50.8). Neither dimorphism is causally involved in inherited expression differences.

The sequence of one human α_2 gene 5′ regulatory region has been reported by Zutter et al.[72,73]. Comparisons with our allele sequences establish that this regulatory region is that of Allele 2. The first 961 bp of the 5′ flanking region of Allele 2 isolated from the K562 cell line directs cell-type specific suppressor and enhancer activity in cells of epithelial origin[72]. This region contains critical elements that are required for efficient expression of this TATA-less promoter. The core promoter (-92/-30) contains two Sp1 elements that are necessary for full transcription activity in erythro-megakaryocytic cell line K562[74]. The distal 5′ region contains a strong megakaryocytic enhancer located at −1655/−1426 that requires two tandem AP1 binding sites[75]. Interestingly, a GATA site and AP2 elements located in this enhancer region and a GATA site within the proximal 961 bp promoter do not mediate enhancer activity in hematopoietic cells. We have recently identified two natural dimorphisms within the human α_2 promoter, $C_{-52}T$ and $C_{-92}G$, which also influence transcription rate[69] and are not linked to alleles 1 or 2. Together with the allelic differences, these promoter dimorphisms contribute to the variability in expression of human $\alpha_2\beta_1$.

Clinical relevance of α_2 polymorphisms

Platelet adhesion to collagen-coated surfaces in whole blood under flow conditions is mediated by both von Willebrand factor (VWF)-dependent recruitment of the platelet glycoprotein Ib–IX receptor complex and collagen interaction with the integrin $\alpha_2\beta_1$. In type 1 von Willebrand disease (VWD), platelet adhesive functions are impaired

Cambridge Books Online © Cambridge University Press, 2009

Integrin subunit α_2 alleles

	Nucleotide residue						
	807	2531	505	799	f	HPA-	a.k.a.
1	**T**	C	Glu	Thr	0.036	5a 12aw	Br[a]
2	C	C	Glu	Thr	0.056	5a 12aw	Br[a]
3	C	C	**Lys**	Thr	0.076	**5b** 12aw	**Br[b]**
4	C	T	Glu	**Met**	<0.001	5a **12bw**	Br[a] **Sit[a](+)**

Fig. 50.8. Integrin α_2 Alleles. Four alleles are defined by combinations of a variety of linked dimorphisms. C/T base substitutions at nucleotide positions 807 and 2531 are shown. A glutamate/lysine substitution at residue 505 generates the Br[a] and Br[b] alloantigens, respectively, while a methionine for threonine substitution at residue 799 gives rise to the Sit[a] alloantigen. Abbreviations: a.k.a, also known as; f, gene frequency; and HPA, human platelet antigen. Gene frequencies for a typical Caucasian population are indicated.

Increased platelet $\alpha_2\beta_1$ density (α_2 Allele 1): a risk factor?

Yes	Odds ratio	No
Myocardial infarction		
1. Moshfegh K. 1999a	3.3	1. Croft S.A. 1999
2. Santoso S. 1999	2.6	
3. Roest M. 2000	2.2	
CVD/stroke		
1. Carlsson L.E. 1999a	3.0	1. Corral J. 1999a
2. Sacchi E. 1999 abstr	3.0	
3. Reiner A.P. 2000	2.0	
Diabetic retinopathy		
1. Matsubara Y. 1999 abstr	3.4	
Coronary artery disease		
		1. Santoso S. 1999
		2. Corral J. 1999a
DVT/venous thrombosis		
		1. Corral J. 1999a
		2. Hessner M.J. 1999
		3. Carlsson L.E. 1999b abstr

Fig. 50.9. Correlation between inheritance of the integrin α_2 Allele 1 (increased $\alpha_2\beta_1$ density) and risk for adverse outcomes in various thrombotic disorders. Individual studies that confirm (YES) or refute (NO) a correlation between inheritance of α_2 allele 1 (807T genotype) and risk for thrombotic disease are indicated. The clinical syndromes include: acute myocardial infarction, cerebral vascular disease (CVD) / stroke, coronary artery disease, deep vein thrombosis (DVT), diabetic retinopathy, and venous thrombosis. Data are tabulated as of November 2000.

due to the decrease in VWF levels in plasma and platelets. Di Paola et al.[76] have demonstrated that the low-density α_2 allele 2 increases the risk factor for bleeding in type I von Willebrand Disease. They measured the frequencies of α_2 alleles in symptomatic patients with five types of VWD (type 1, $n=78$; type 2A, $n=25$, type 2B, $n=14$; type 2M, $n=10$; and type 3, $n=20$). Compared to the normal group, no significant difference in allele frequencies was observed among individuals with types 2A, 2B, 2M, or 3 VWD. However, the frequency of allele 2 among type 1 VWD patients ($=0.71$) was significantly higher than that of the normal population ($P=0.007$). Also, in patients with VWD type 1 and borderline to normal ristocetin-cofactor (VWF: RCo) activity values, collagen receptor density correlates inversely with closure time in a high shear stress system, the platelet function analyzer (PFA-100; Dade-Behring). It was proposed that low platelet $\alpha_2\beta_1$ density results in less efficient primary platelet adhesion and may result in increased tendency to bleed, as evidenced by the high frequency of this polymorphism in patients with type 1 VWD compared with normal individuals. In addition, this may account for the variability between patients with similar levels of VWF antigen, but strikingly different bleeding histories.

Santoso et al.[77] investigated the relationship of the α_2 alleles to the risk of coronary artery disease (CAD) and myocardial infarction (MI). An allele-specific polymerase chain reaction (PCR) was developed for genotyping α_2 alleles. DNA samples from 2237 male patients who underwent coronary angiography on account of coronary heart disease as verified illness or presumptive diagnosis were genotyped. They found a strong association between allele 1 and non-fatal MI among individuals younger than the mean age of 62 years ($n=1057$; odds ratio, 1.57; $P=0.004$) and an even stronger association among individuals within the youngest 10% of the study sample (<49 years; $n=223$; odds ratio, 2. 61; $P=0.009$). In contrast, no evidence of an association between α_2 alleles and CAD was found. It was concluded that inherited platelet $\alpha_2\beta_1$ variations might have an important impact on acute thrombotic disease in younger individuals. This has been confirmed by a number of other investigators (Fig. 50.9)[78,79]. In addition, a significant correlation has been found between the expression of Allele 1 and risk for adverse outcomes in younger individuals with cerebral vascular disease or stroke [80–82] and diabetic retinopathy[83].

In contrast, the density of platelet $\alpha_2\beta_1$ has not been found to be a risk factor for venous thrombosis[84–86] (Fig. 50.9). This lack of influence on thrombosis on the venous side is a typical finding for all of the platelet glycoprotein dimorphisms that are currently under study.

Cambridge Books Online © Cambridge University Press, 2009

Hormonal regulation of $\alpha_2\beta_1$ expression

Gender differences figure prominently in risk for thromboembolic disease, leading to the suspicion that steroid hormones might influence the expression and or activity of key platelet glycoprotein receptors. It has been known for some time that hormone replacement therapy (HRT) in postmenopausal women can predispose to venous thrombosis, but it is only recently that a potential influence of HRT on platelets in the coronary circulation has been proposed[87,88]. Tarantino et al.[87] observed that, in women, platelet adhesion to type I collagen shows a biphasic periodicity in synchrony with the menstrual cycle and that human megakaryocytes express the estrogen receptor (ER). This finding implies that platelet collagen receptor function may be modulated by sex hormone levels and would be consistent with the known correlation between ER and $\alpha_2\beta_1$ expression in certain cell lineages[72,89]. Khetawat et al.[88] have subsequently observed that human megakaryocytes contain mRNA specific for the ER β and the androgen receptor (AR), and that the transcripts of both can be located in the platelet cytoplasm. Megakaryocytes are able to respond in a regulated manner to testosterone. These reports provide compelling evidence that sex hormones may be able to mediate gender differences in platelet function and antigenicity. It remains to be determined to what extent the effects of these hormones occur through genomic or non-genomic pathways.

Platelet GPVI

GPVI is a major platelet glycoprotein (60–65 kDa) (Fig. 50.10) that had been considered a putative receptor for collagen since the identification of a patient with a mild bleeding disorder whose platelets lacked GPVI and exhibited defective collagen-induced responses[90–92]. Collagen binding to GPVI induces platelet activation through a pathway that involves phosphorylation of the FcRγ chain followed by the binding of Syk and the phosphorylation-dependent activation of PLCγ2[93–96]. The sequence of collagen that is recognized by GPVI has been identified as Gly–Pro–Hyp, and a synthetic collagen-related peptide (CRP) based on the triple helical form of this tripeptide sequence is a GPVI-specific platelet agonist[97]. The GPVI–Fcγ complex will transduce outside-in signals by an immune receptor-like mechanism that involves p72^{SYK} activation, results in PLCγ2 activation, and leads to release of granule contents and platelet aggregation[98]. The C-type lectin, convulxin (CVX), from the tropical rattlesnake, *Crotalus durissus terrificus*, is a multimeric protein that binds specifically to GPVI and induces platelet activation

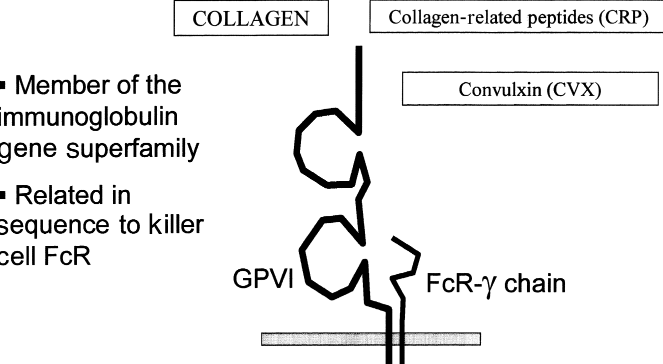


Fig. 50.10. Platelet glycoprotein VI. Platelet glycoprotein VI is a member of the immunoglobulin gene superfamily and is related in sequence to members of the killer cell FcR group. GPVI is non-covalently associated with, and requires for expression, the FcR γ chain. Both proteins contain transmembrane regions and cytoplasmic domains. GPVI binds specifically to collagens, specific sequences within type I collagen typified by the collagen-related peptide (CRP) and the snake venom protein convulxin (CVX).

through the clustering of GPVI [99,100]. CVX is a ready tool for the detection and quantitation of GPVI that also proved invaluable for the isolation and eventual sequencing of this receptor[101].

GPVI cDNA has an open reading frame of 1017 base pairs coding for a protein of 339 amino acids including a putative 23-amino acid signal sequence and a 19-amino acid transmembrane domain between residues 247 and 265. By sequence homology, GPVI is a member of the immunoglobulin superfamily, and its sequence is closely related to FcαR and the natural killer receptors. It contains two Ig-C2-like domains formed by disulfide bridges. The cytoplasmic tail, consisting of 51 amino acids, is atypical of, and shows little homology with, the C-terminal part of the other members of this protein family.

Polymorphic expression of GPVI

In the initial characterizations of platelet GPVI function, some degree of heterogeneity was noted among apparently normal subjects. For example, Jandrot-Perrus and coworkers[100] had reported that, among normal healthy volunteers, purified convulxin induced platelet aggregation at levels between 15 and 35 pM, but they noted a significant heterogeneity in platelet sensitivity to convulxin. Moreover, variability in ligand binding to GPVI was also evident in the findings of Polgar et al.[99].

Cambridge Books Online © Cambridge University Press, 2009

To specifically investigate this potential heterogeneity, we developed a semiquantitative ligand blot assay in which biotin-conjugated convulxin binds selectively to GPVI among the total content of platelet proteins separated by SDS–polyacrylamide gel electrophoresis. We found a fivefold range of platelet GPVI content among twenty-three normal healthy subjects (K. Furihata and T.J. Kunicki, unpublished observations). In addition, we found that prothrombinase activity induced by CVX- or CRP, but not ionophore A23187, is directly proportional to the platelet content of GPVI ($P<0.001$). The fivefold variation in GPVI content is strikingly similar to the previously established range in expression of platelet integrin $\alpha_2\beta_1$ (see above). Among the donors in this study, there was a direct correlation between platelet $\alpha_2\beta_1$ density and GPVI content ($P=0.004$). In view of the well-documented association of GPVI with platelet procoagulant activity, this variation in GPVI content may represent yet another genetically controlled risk factor predisposing individuals to hemorrhagic or thromboembolic disorders.

At this time, however, a genetic basis for variation in GPVI content has not been defined. The possibilities that either the two collagen receptors are physically associated or that the expression of the two receptors is coordinately regulated need to be addressed. In either case, the variation in GPVI content might be driven by the already defined genetic regulation in expression of the integrin $\alpha_2\beta_1$.

Stage 3. Platelet cohesion: thrombus formation mediated by integrin $\alpha_{IIb}\beta_3$

Platelet integrin $\alpha_{IIb}\beta_3$ (GPIIb–IIIa)

The megakaryocyte-specific integrin, α_{IIb}, is the α-subunit of the $\alpha_{IIb}\beta_3$ complex (Fig. 50.11). In the α_{IIb} gene promoter, there are two GATA-binding elements, one of which (at −454) is most critical for transcription[102–104]. A potential negative regulatory element is located within −200/−100 in both the rat and human genes[50,105]. A GA-rich region encompassing −14 appears to be a non-consensus Sp1-binding site that interacts with an Ets-consensus site at approximately −34[106]. This promoter provides another example of the cooperative effect of Ets-like proteins and Sp1 in transcriptional activation of TATA-less genes. The human α_{IIb} and β_3 genes are both physically located on chromosome 17 (17q21.32), but a detailed map of the α_{IIb} region suggests that the αIIb and β3 genes may be ≥1 mb apart, a separation that is too great to consider them located within a single tissue-specific locus[107].

FOG, the cofactor for GATA-1, plays a key role in normal erythroid and megakaryocytic differentiation[37]. FOG is a multitype zinc finger protein that binds to the amino-terminal finger of GATA-1 and modulates the transcription of GATA-1 target genes. FOG acts in concert with GATA-1 and possibly GATA-2 to stimulate α_{IIb} expression[108].

Loss of FOG in mice knockout experiments leads to the elimination of the megakaryocytic lineage at a very early stage, contrasting with the late blockage of megakaryocytic differentiation occurring with GATA-1[109]. This finding suggests that, although acting as an essential cofactor of GATA-1 in erythroid cells, FOG may act independently from GATA-1 in megakaryocytes.

The DNA *cis*-acting elements of gene promoters are also important for regulation of megakaryocyte gene expression. Transcription is regulated by the differential engagement of cis elements in patterns that are specific to each cell lineage. For example, most of the megakaryocytic gene promoters contain GATA and Ets binding sites, and the engagement of these is crucial for transcriptional control and lineage restriction of these genes[9,103,110,111].

Cumulative evidence has led to a model in which α_{IIb} gene transcription is switched off in non-megakaryocytic cells but kept active throughout megakaryocyte differentiation[50]. The α_{IIb} gene enhancer that bears GATA and Ets elements is active in both erythroid and megakaryocytic cells[50,104], and other positive *cis*-active elements that control α_{IIb} are also present in other cell lineages. Thus, the mechanism whereby α_{IIb} gene transcription is restricted to the megakaryocyte lineage must lie elsewhere. One explanation is that the positive elements are overridden by the action of a strong repressor that is lineage restricted. This repressor is even active in megakaryocytic cells, where it attenuates α_{IIb} gene transcriptional activity to a medium level. In other cell lineages, the repressor is even more active, and α_{IIb} gene transcription is essentially blocked. The expression of $\alpha_{IIb}\beta_3$ by avian multilineage hematopoietic cells suggests that, although detected in early hematopoietic progenitors, α_{IIb} expression is turned off in the course of progenitor commitment[112]. At the same time, α_{IIb} expression increases during the differentiation of megakaryocytes[113–115].

A comparison of the effect of a heterologous environment on the activity of the human and murine α_{IIb} promoter has shed some light on the transcriptional control of this gene. When transfected in a murine context, the human α_{IIb} promoter preserves its megakaryocytic specificity[116]. The murine α_{IIb} promoter is active and megakaryocyte specific in murine cell lines. However, it loses its lineage restriction when transfected into the human K562 cell line, where it exhibits an even higher expression

Cambridge Books Online © Cambridge University Press, 2009

level than in HEL cells. Conversely, the human α_{IIb} promoter is nearly inactive in K562 cells. The murine promoter was inactive in KG1 cells and in HeLa cells, indicating that the deregulation observed with this promoter only affects the erythro-megakaryocytic system[116].

The murine α_{IIb} promoter contains an enhancer element homologous to the erythro-megakaryocytic human enhancer. Recall that the activity of the human α_{IIb} promoter is dependent on two essential GATA and Ets elements. In the murine promoter, the erythro-megakaryocytic enhancer activity is dependent on the −456 GATA site but not on the −505 Ets element[116]. This result is different from the concerted engagement of both elements required for full activity of the human enhancer.

In addition to GATA-1, the murine −456 GATA formed an additional complex (termed B) of higher molecular weight[116]. The B complex was observed in all nuclear extracts tested, including HeLa cells, suggesting B complex is ubiquitously expressed. B complex did not interact with anti-GATA-1 antibodies, indicating it is different from GATA-1. Human and the murine promoters differ significantly in that they probably use different GATA-1 partners. The enhancer Ets binding site shown to be crucial for the human enhancer activity is inactive in the murine enhancer. Perhaps in the case of the mouse α_{IIb} promoter, GATA-1/B complex interactions may be an alternative to GATA-1/ETS cooperation for the human α_{IIb} promoter in human cell lines[116]. Although apparently not required for human α_{IIb} transcriptional regulation in human cell lines, B complex is present and active in these cells, suggesting that it could regulate other sets of human genes.

The integrin $\alpha_{IIb}\beta_3$

The numerically predominant platelet integrin $\alpha_{IIb}\beta_3$ (Fig. 50.11) mediates the common cohesive pathway that results from platelet activation in vivo, i.e. platelet aggregation supported by the binding of adhesive proteins, such as fibrinogen and von Willebrand factor (VWF).

Three allelic variants of the integrin subunit α_{IIb} (Fig. 50.12) have been defined which differ at either residue 837 (Val or Met)[117] or residue 843 (Ser or Ile)[118]. A very complex scenario occurs with the integrin subunit β_3. Eight different alleles of the β_3 can be distinguished (Fig. 50.13) which differ at seven positions within the coding sequence. These dimorphic residues and their serologic designations are: Leu_{33}/Pro_{33} (HPA–1a/HPA–1b)[119]; Arg_{62}/Gln_{62} (HPA–10wa/HPA–10wb)[120]; Arg_{143}/Gln_{143} (HPA–4a/HPA–4b)[121]; Pro_{407}/Ala_{407} (HPA–7a/HPA–7b)[122]; Arg_{489}/Gln_{489} (HPA–6a/HPA–6b)[123]; and Arg_{636}/Cys_{636} (HPA–8a/HPA–8b)[124]. The rare HPA–1b allele characterized by the presence of Arg_{40} does not appear to be serologically distinguishable from the HPA–1b allele bearing the more common Leu_{40}[125].

Fig. 50.11. Integrin $\alpha_{IIb}\beta_3$. (Left panel) This receptor is a heterodimer composed of noncovalently associated α_{IIb} and β_3 subunits. The α_{IIb} subunit is a two chain molecule composed of a larger chain disulfide-bonded to a smaller chain, the latter bearing a transmembrane region and cytoplasmic domain. The β_3 subunit is a single chain polypeptide with a transmembrane region and cytoplasmic domain. (Right panel) An important dimorphism of the β_3 subunit results from a leucine/proline substitution at residue 33 within a small disulfide-looped region, giving rise to the Pl^{A1} and Pl^{A2} alloantigens, respectively.

Integrin subunit α_{IIb} alleles

Amino acid 837	843	f	HPA-	a.k.a
Val	Ile	0.61	3a 9a	Bak^a, Lek^a
Val	Ser	0.36	3b 9a	Bak^b
Met	Ser	0.03	3b 9b	Bak^b, Max^a

Fig. 50.12. Integrin subunit α_{IIb} alleles. Three alleles are generated by a valine/methionine substitution at residue 837 and an isoleucine/serine substitution at residue 843. These give rise to the Bak (Lek) and Max alloantigen systems, respectively. Abbreviations: a.k.a, also known as: f, gene frequency; and HPA, human platelet antigen. Gene frequencies for a typical Caucasian population are indicated.

Clinical relevance of $\alpha_{IIb}\beta_3$ polymorphisms

The first receptor to be scrutinized was $\alpha_{IIb}\beta_3$. In a pioneering study in 1996, Weiss et al.[126] reported that the gene frequency of the β_3 Pl^{A2} (Pro_{33}) allele was 3.6 times higher

Cambridge Books Online © Cambridge University Press, 2009

Integrin subunit β_3 alleles

Amino acid residue									
33	40	62	143	407	489	636	f	PlA2	HPA-
Leu	Leu	Arg	Arg	Pro	Arg	Arg	0.85		1a 10a 4a 7a 6a 8a
Pro	Leu	Arg	Arg	Pro	Arg	Arg	0.15	PlA2	1b 10a 4a 7a 6a 8a
Pro	**Arg**	Arg	Arg	Pro	Arg	Arg	0.005	PlA2	1b 10a 4a 7a 6a 8a
Leu	Leu	**Gln**	Arg	Pro	Arg	Arg	<0.001		1a 10b 4a 7a 6a 8a
Leu	Leu	Arg	**Gln**	Pro	Arg	Arg	<0.01		1a 10a 4b 7a 6a 8a
Leu	Leu	Arg	Arg	**Ala**	Arg	Arg	<0.001		1a 10a 4a 7b 6a 8a
Leu	Leu	Arg	Arg	Pro	**Gln**	Arg	<0.001		1a 10a 4a 7a 6b 8a
Leu	Leu	Arg	Arg	Pro	Arg	**Cys**	<0.001		1a 10a 4a 7a 6a 8b

Fig. 50.13. Integrin Subunit β_3 Alleles. Eight alleles are generated by dimorphic substitutions at residues 33, 40, 62, 143, 407, 489, and 636. These give rise to the HPA phenotypes listed at the right of the figure. Two alleles are Pl^{A2}-positive (contain proline-33) and are indicated within the boxed area. The remaining six alleles are Pl^{A1}-positive (contain leucine-33). Abbreviations: a.k.a, also known as; f, gene frequency; and HPA, human platelet antigen. Gene frequencies for a typical Caucasian population are indicated.

Expression of the integrin $\beta_3 Pl^{A2}$ allele: a risk factor?

Yes	No
Myocardial infarction (younger individuals)	
1. Weiss E.J. 1996	1. Marian A.J. 1996
2. Carter A.M. 1996	2. Osborn S.V. 1996
3. Zotz R.B. 1998	3. Herrmann S.M. 1997
4. Anderson J.L. 1999	4. Ridker P.M. 1997
5. Mikkelsson J. 1999	6. Samani N.J. 1997
6. Tereshchenko S.N. 1999	7. Durante-Mangoni E. 1998
7. Ardissino D. 1999	8. Gardemann A. 1998
8. Mikkelsson J. 2000	9. Mamotte C.D. 1998
	10. Scaglione L. 1998
	11. Moshfegh K. 1999b abstr
	12. Kekomaki S. 1999
	13. Bottiger C. 2000
Coronary artery disease	
1. Garcia-Ribes M. 1998	1. Corral J. 1997
2. Gardemann A. 1998	2. Mamotte C.D. 1998
	3. Zotz R.B. 1998
	4. Anderson J.L. 1999
	5. Kekomaki S. 1999
	6. Mikkelsson J. 1999
	7. Bottiger C. 2000

Fig. 50.14. Correlation between inheritance of the integrin β_3 Pl^{A2} allele (proline-33) and risk for adverse outcomes in acute myocardial infarction (in younger individuals) or coronary artery disease. In most studies, younger individuals are defined as those ≤62 years old. In most studies, coronary artery disease is present if there is 50% occlusion of at least one major coronary artery. Individual studies that confirm (YES) or refute (NO) a correlation between inheritance of β_3 Pl^{A2} allele and risk for thrombotic disease are indicated. Data are tabulated as of November 2000.

Expression of the integrin $\beta_3 Pl^{A2}$ allele: a risk factor?

Yes	No
Restonosis after stent	
1. Walter D.H. 1997	1. Laule M. 1999
2. Kastrati A. 2000	
Acute renal allograft rejection	
1. Salido E. 1999	
Restonosis after angioplasty	
	1. Mamotte C.D. 1998
	2. Laule M. 1999
CVD/stroke	
1. Carter A.M. 1998	1. Corral J. 1997
	2. Ridker P.M. 1997
	3. Wagner K.R. 1998
	4. Kekomaki S. 1999
Venous thrombosis	
	1. Ridker P.M. 1997

Fig. 50.15. Correlation between inheritance of the integrin β_3 Pl^{A2} allele (proline-33) and risk for adverse outcomes in other thrombotic diseases. Individual studies that confirm (YES) or refute (NO) a correlation between inheritance of β_3 Pl^{A2} allele and risk for thrombotic disease are indicated. The clinical syndromes include: acute renal allograft rejection, cerebral vascular disease (CVD) / stroke, restenosis after angioplasty, restenosis after stent, and venous thrombosis. Data are tabulated as of November 2000.

Cambridge Books Online © Cambridge University Press, 2009

among younger case patients (<60 years of age) with myocardial infarction or unstable angina as compared to age-matched controls (odds ratio = 6.2). This was the first instance of a platelet-specific risk factor in acute coronary thrombosis, and this study was rapidly followed by numerous reports that confirmed or denied this association of Pl^{A2} as a risk factor for acute coronary disease (Fig. 50.14). While many studies have confirmed the association between the Pl^{A2} allele and increased thrombotic risk in myocardial infarction or coronary artery disease, a substantial number have refuted this observation. Other clinical settings where risk might be involved are restenosis after stent[127,128] or acute renal allograft rejection (Fig. 50.15). On the other hand, as was the case with the genetic differences in integrin α_2, no correlation between integrin $\alpha_{IIb}\beta_3$ and risk for venous thrombosis has yet been observed (Fig. 50.15).

After the β_3 Pro_{33} allele had been implicated in risk for thrombosis, the search began for any indication that this allele might confer increased biological activity upon the integrin $\alpha_{IIb}\beta_3$. Although most of the identifed differences have not been obvious, they *are* statistically significant and suggest that this allele confers a lower threshold for agonist-induced platelet responses. Most recently, Vijayan et al.[130] showed, in transfected CHO or 293 cells, that the Pl^{A2} allele exhibited increased binding to immobilized fibrinogen, but not to soluble fibrinogen or immobilized fibronectin. In addition, Michelson et al.[131] found that Pl^{A2}-positive platelets displayed a lower threshold for activation and an increased sensitivity to antiplatelet drugs (e.g. aspirin and abciximab).

References

1 Tasi S-F, Masrtin DIK, Zon LI, D'Andrea AD, Wong GG, Orkin SH. Cloning of cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells. *Nature* 1989; 339: 446–451.

2 Martin DI, Zon LI, Mutter G, Orkin SH. Expression of an erythroid transcription factor in megakaryocytic and mast cell lineages. *Nature* 1990; 344: 444–447.

3 Romeo PH, Prandini M-H, Joulin V et al. Megakaryocytic and erythrocytic lineages share specific transcription factors. *Nature* 1990; 344: 447–449.

4 Zon LI, Yamaguchi Y, Yee K et al. Expression of mRNA for the GATA-binding proteins in human eosinophils and basophils: potential role in gene transcription. *Blood* 1993; 81[12]: 3234–3241.

5 Dorfman DM, Wilson DB, Bruns GA, Orkin SH. Human transcription factor GATA-2. Evidence for regulation of preproendothelin-1 gene expression in endothelial cells. *J Biol Chem* 1992; 267: 1279–1285.

6 Orkin SH. Transcription factors and hematopoietic development. *J Biol Chem* 1995; 270: 4955–4958.

7 George KM, Leonard MW, Roth ME et al. Embryonic expression and cloning of the murine GATA-3 gene. *Development* 1994; 120: 2673–2686.

8 Kornhauser JM, Leonard MW, Yamamoto M. Temporal and spatial changes in GATA transcription factor expression are coincident with development of the chicken optic tectum. *Brain Res Mol Brain Res* 1994; 23: 100–110.

9 Bastian LS, Yagi M, Chan C et al. Analysis of the megakaryocyte glycoprotein IX promoter identifies positive and negative regulatory domains and functional GATA and Ets sites. *J Biol Chem* 1996; 271: 18554–18560.

10 Visvader J, Adams JM. Megakaryoytic differentiation induced in 416B myeloid cells by GATA-2 and GATA-3 transgenes or 5-azacytidine is tightly coupled to GATA-1 expression. *Blood* 1993; 82: 1493–1501.

11 Pevny L, Simon MC, Robertson E et al. Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* 1991; 349: 257–260.

12 Pevny L, Lin CS, D'Agati V, Simon MC, Orkin SH, Costantini F. Development of hematopoietic cells lacking transcription factor GATA-1. *Development* 1995; 121: 163–172.

13 Fujiyoshi Y, Browne CP, Cunnif K, Goff SC, Orkin SH. Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1. *Proc Natl Acad Sci USA* 1996; 93: 12355–12358.

14 Weiss MJ, Orkin SH. Transcription factor GATA-1 permits survival and maturation of erythroid precursors by preventing apoptosis. *Proc Natl Acad Sci USA* 1995; 92[21]: 9623–9627.

15 Shivdasani RA, Fujiwara Y, Mcdevitt MA, Orkin SH. A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development *EMBO J* 1997; 16: 3965–3973.

16 Weiss MJ, Orkin SH. GATA transcription factors; key regulators of hematopoiesis. *Exp Hematol* 1995; 23: 99–107.

17 Leprince D, Gegonne A, Coll J et al. A putative second cell-derived oncogene of the avian leukaemia retrovirus E26. *Nature* 1983; 306: 395–397.

18 Seth A, Ascione R, Fisher RJ, Mavrothalassitis GJ, Bhat NK, Papas TS. The ets gene family. *Cell Growth Differ* 1992; 3: 327–334.

19 Athanasiou M, Clausen PA, Mavrothalassistis GJ, Zhang XK, Watson DK, Blair DG. Increased expression of the ETS-related transcription factor FLI-1/ERGB coorelates with and can induce the megakaryocytic phenotype. *Cell Growth Differ* 1996; 7: 1525–1534.

20 Moreau-Gachelin F, Wendling F, Molina T et al. Spi-1/PU.1transgenic mice develop multistep erythroleukemias. *Mol Cell Biol* 1996; 16: 2453–2463.

21 Yamada T, Kihara–Negishi F, Yamamoto H, Yamamoto M, Hashimoto Y, Oikawa T. Reduction of DNA binding activity of

Cambridge Books Online © Cambridge University Press, 2009

the GATA-1 transcription factor in the apoptotic process induced by overexpression of PU.1 murine erythroleukemia cells. *Exp Cell Res* 1998; 245: 186–194.
22 Kenny D, Gill JC, Montgomery RR. The critical interaction of glycoprotein (GP) lb beta with GPIX – a genetic cause of Bernard–Soulier Syndrome. *Blood* 1999; 9: 2968–2975.
23 Margaglione M, D'Andrea G, Grandone E, Brancaccio V, Amoriello A, Di Minno G. Compound heterozygosity (554-589 del, C^{515}-T transition) in the platelet glycoprotein lbα gene in a patient with a severe bleeding tendency. *Thromb Haemost* 1999; 81: 486–492.
24 Kenny D, Jonsson OG, Morateck PA, Montgomery RR. Naturally occurring mutations in glycoprotein Ibalpha that result in defective ligand binding and synthesis of a truncated protein. *Blood* 1998; 92: 175–183.
25 Lopez JA, Andrews RK, Afshar-Kharghan V, Berndt MC. Bernard–Soulier syndrome. *Blood* 1998; 91: 4397–4418.
26 Moran N, Morateck PA, Deering A et al. Surface expression of glycoprotein Ib alpha is dependent on glycoprotein Ib beta: evidence from a novel mutation causing Bernard–Soulier syndrome. *Blood* 2000; 96: 532–539.
27 Strassel C, Lanza F, de la Salle C et al. A novel missense mutation in GP Ibbeta prevents the normal maturation and surface expression of the GPIb–IX–V complex in a patient with the Bernard–Soulier syndrome. *Blood* 2000; 96: 816a–816a.
28 Yagi M, Edelhoff S, Disteche CM, Roth GJ. Structural characterization and chromosomal location of the gene encoding human platelet glycoprotein Ib beta. *J Biol Chem* 1994; 269: 17424–17427.
29 Wenger RH, Kieffer N, Wicki AN, Clemetson KJ. Structure of the human blood platelet membrane glycoprotein lb alpha gene. *Biochem Biophys Res Commun* 1988; 156: 389–395.
30 Wenger RH, Wicki AN, Kieffer N, Adolph S, Hameister H, Clemetson KJ. The 5′-flanking region and chromosomal localization of the gene encoding human platelet membrane glycoprotein Ibalpha. *Gene* 1989; 85: 517–524.
31 Kelly MD, Essex DW, Shapiro SS et al. Complementary DNA cloning of the alternatively expressed endothelial cell glycoprotein Ibβ (GPlbβ) and localization of the GPlbβ gene to chromosome 22. *J Clin Invest* 1994; 93: 2417–2424.
32 Yagi M, Edelhoff S, Disteche CM, Roth GJ. Human platelet glycoproteins V and IX: mapping of two leucine rich glycoprotein genes to chromosome 3 and analysis of structures. *Biochemistry* 1995; 34: 16132–16137.
33 Michelson AD, Loscalzo J, Melnick B, Coller BS, Handin RI. Partial characterization of a binding site for von Willebrand factor on glycocalicin. *Blood* 1986; 67: 19–26.
34 Takamatsu J, Horne MK, Gralnick HR. Identification of the thrombin receptor on human platelets by chemical crosslinking. *J Clin Invest* 1986; 77: 362–368.
35 Du X, Fox JE, Pei S. Identification of a binding sequence for the 14-3-3 protein within the cytoplasmic domain of the adhesion receptor, platelet glycorprotein IBalpha. *J Biol Chem* 1996; 271: 7362–7367.
36 Meyer SC, Zuerbig S, Cunningham CC, Hartwig JH, Bissell T, Gardner K et al. Identification of the region in actin-binding protein that binds to the cytoplasmic domain of glycoprotein Ibalpha. *J Biol Chem* 1997; 272: 2914–2919.
37 Tsang AP, Visvader JE, Turner CA et al. FOG, a multitype zinc finger protein, acts as a cofactor for transcription factor GATA-1 in erythroid and megakaryocytic differentiation. *Cell* 1997; 90: 109–119.
38 Hashimoto Y, Ware J. Identification of essential GATA and Ets binding motifs within the promoter of the platelet glycoprotein Ib alpha gene. *J Biol Chem* 1995; 270: 24532–24539.
39 Ludlow LB, Schick BP, Budarf ML et al. Identification of a mutation in a GATA binding site of the platelet glycoprotein Ib beta promoter resulting in the Bernard–Soulier syndrome. *J Biol Chem* 1996; 271: 22076–22080.
40 Lopez JA, Ludwig EH, McCarthy BJ. Polymorphism of human glycoprotein Ibα results from a variable number of tandem repeats of a 13-amino acid sequence in the mucin-like macroglycopeptide region. *J Biol Chem* 1992; 267: 10055–10061.
41 Ishida F, Furihata K, Ishida K et al. The largest variant of platelet glycoprotein Ibα has four tandem repeats of 13 amino acids in the macroglycopeptide region and a genetic linkage with Methionine[145]. *Blood* 1995; 86: 1356–1360.
42 Murata M, Matsubara Y, Kawano K et al. Coronary artery disease and polymorphisms in a receptor mediating shear stress-dependent platelet activation. *Circulation* 1997; 96: 3281–3286.
43 Gonzalez-Conejero R, Lozano ML, Rivera J et al. Polymorphisms of platelet membrane glycoprotein Ib alpha associated with arterial thrombotic disease. *Blood* 1998; 92: 2771–2776.
44 Kuijpers RWAM, Faber NM, Cuypers HTM, Ouwehand EH, Von dem Borne AEGK. NH_2-terminal globular domain of human platelet glycoprotein Ibα has a methionine[145]/threonine[145] amino acid polymorphism, which is associated with the HPA-2 (Ko) alloantigens. *J Clin Invest* 1992; 89: 381–384.
45 Murata M, Furihata K, Ishida F, Russell SR, Ware J, Ruggeri ZM. Genetic and structural characterization of an amino acid dimorphism in glycoprotein Ibα involved in platelet transfusion refractoriness. *Blood* 1992; 79: 3086–3090.
46 Afshar-Khargan V, Li CQK-AM, Lopez JA. Polymorphism of the platelet glycoprotein (GP) Ib Kozak sequence determines the surface level of the GP Ib–IX–V complex and risk for early myocardial infarction. *Blood* 1999; 94: 186–191.
47 Kaski S, Kekomaki R, Partanen J. Systematic screening for genetic polymorphism in human platelet glycoprotein Ib alpha. *Immunogenetics* 1996; 44: 170–176.
48 Dong JF, Gao S, Lopez JA. Synthesis assembly, and intracellular transport of the platelet glycoprotein Ib–IX–V complex. *J Biol Chem* 1998; 273: 31449–31454.
49 Sachs UJH, Kiefel V, Bohringer M, Afshar-Kharghan V, Kroll H, Santoso S. Single amino acid substitution in human platelet glycoprotein Ib beta is responsible for the formation of the platelet-specific alloantigen Iy[a]. *Blood* 2000; 95: 1849–1855.

Cambridge Books Online © Cambridge University Press, 2009

50 Prandini MH, Martin F, Thevenon D, Uzan G. The tissue-specific transcriptional regulation of the megakaryocytic glycoprotein IIb gene is controlled by interactions between a repressor and positive *cis*-acting elements. *Blood* 1996; 88: 2062–2070.
51 Dong JF, Sae-Tung G, Lopez JA. Role of glycoprotein V in the formation of the platelet high-affinity thrombin-binding site. *Blood* 1997; 89: 4355–4363.
52 Ramakrishnan V, Reeves PS, DeGuzman F et al. Increased thrombin responsiveness in platelets from mice lacking glycoprotein V. *Proc Natl Acad Sci USA* 1999; 96: 13336–13341.
53 Lepage A, Uzan G, Touche N et al. Functional characterization of the human platelet glycoprotein V gene promoter: A specific marker of late megakaryocytic differentiation. *Blood* 1999; 19: 3366–3380.
54 Fox JE. Linkage of a membrane skeleton to integral membrane glycoproteins in human platelets. Identification of one of the glycoproteins as glycoprotein Ib. *J Clin Invest* 1985; 76: 1673–1683.
55 Okita JR, Pidard D, Newman PJ, Montgomery RR, Kunicki TJ. On the association of glycoprotein Ib and actin-binding protein in human platelets. *J Cell Biol* 1985; 100: 317–321.
56 Du X, Harris SF, Tetaz TJ, Ginsberg MJ, Berndt MC. Association of a phospholipase A2 (14-3-3 protein) with the platelet glycoprotein IB–IX complex. *J Biol Chem* 1994; 269: 18287–18290.
57 Kroll MH, Harris TS, Moake JL, Handin RI, Schafer AI. von Willebrand Factor binding to platelet GPIb initiates signals for platelet activation. *J Clin Invest* 1991; 88: 1568–1573.
58 Razdan K, Hellums JD, Kroll NH. Shear-stress-induced von Willebrand factor binding to platelets causes the activation of tyrosine kinase(s). *Biochem J* 1994; 302: 681–686.
59 Ikeda Y, Handa M, Kamata T et al. Transmembrane calcium influx associated with von Willebrand factor binding to GP Ib in the initiation of shear-induced platelet aggregation. *Thromb Haemost* 1993; 69: 496–502.
60 Jackson SP, Schoenwaelder SM, Yuan Y, Rabinowitz I, Salem HH, Mitchell CA. Adhesion receptor activation of phosphatidylinositol 3-kinase, von Willebrand factor stimulates the cytoskeletal association and activation of phosphatidylinositol 3-kinase and pp60c-src in human platelets. *J Biol Chem* 1994; 269: 27093–27099.
61 Asazuma N, Ozaki Y, Satoh K, Yatomi Y, Handa M, Fujimara Y et al. Glycoprotein Ib–von Willebrand factor interactions activate tyrosine kinases in human platelets. *Blood* 1997; 90: 4789–4798.
62 Muslin AJ, Tanner JW, Allen PM, Shaw AS. Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell* 1996; 84: 889–897.
63 Falati S, Edmead CE, Poole AW. Glycoprotein IB–V–IX, a receptor for von Willebrand factor, couples physically and functionally to the Fc receptor gamma-chain Fyn, and Lyn to activate human platelets. *Blood* 1999; 94: 1648–1656.
64 Santoso S, Zimmerman P, Sachs UJH, Gardemann A. The Kozak sequence polymorphism of the glycoprotein IBalpha gene is associated with the extent of coronary artery disease in younger patients. *Blood* 2000; 96[11]: 624a.
65 Peterson ET, Sutherland R, Robinson DL et al. An integrated physical map for the short arm of human chromosome 5. *Genome Res* 1999; 9: 1250–1267.
66 Takada Y, Hemler ME. The primary structure of the VLA-2/collagen receptor alpha 2 subunit (platelet GPIa): Homology to other integrins and the presence of a possible collagen-binding domain. *J Cell Biol* 1989; 109: 397–407.
67 Kunicki TJ, Kritzik M, Annis DS, Nugent DJ. Hereditary variation in platelet integrin $\alpha_2\beta_1$ copy number is associated with two silent polymorphisms in the α_2 gene coding sequence. *Blood* 1997; 89: 1939–1943.
68 Kritzik M, Savage B, Nugent DJ, Santoso S, Ruggeri ZM, Kunicki TJ. Nucleotide polymorphisms in the alpha 2 gene define multiple alleles which are associated with differences in platelet alpha 2 beta 1. *Blood* 1998; 92: 2382–2388.
69 Jacquelin B, Tarantino M, Kritzik M, Rozenshteyn D, Koziol JA, Kunicki TJ. Allele-dependent transcriptional regulation of the human integrin alpha 2 gene. *Blood* 2000; in press.
70 Santoso S, Kalb R, Walka V, Kiefel V, Mueller-Eckhardt C, Newman PJ. The human platelet alloantigens Br(a) and Br(b) are associated with a single amino acid polymorphism on glycoprotein Ia (integrin subunit α2). *J Clin Invest* 1993; 92: 2427–2432.
71 Santoso S, Amrhein J, Sachs U, Walka MM, Kroll H, Kiefel V. A mutational hot spot $Thr_{799}Met$ on the $?_2$ integrin subunit leads to the formation of new human platelet alloantigen Sit^a and affects collagen-induced aggregation. *Blood* 1997; 90: 261a.
72 Zutter MM, Santoro SA, Painter AS, Tsung YL, Gafford A. The human α_2 integrin gene promoter. Identification of positive and negative regulatory elements important for cell-type and developmentally restricted gene expression. *J Biol Chem* 1994; 269: 463–469.
73 Zutter MM, Painter AA, Saatz WD, Tsung YL. Regulation of α_2 integrin gene expression in cells with megakaryocytic features: a common theme of three necessary elements. *Blood* 1995; 86: 3006–3014.
74 Zutter MM, Ryan EE, Painter AD. Binding of phosphorylated Sp1 protein to tandem Sp1 binding sites regulates alpha2 integrin gene core promoter activity. *Blood* 1997; 90: 678–689.
75 Zutter MM, Painter AD, Yang X. The megakaryocyte/platelet-specific enhancer of the alpha 2 beta 1 integrin gene: two tandem AP1 sites and the mitogen-activated protein kinase signaling cascade. *Blood* 1999; 93: 1600–1611.
76 Di Paola J, Federici AB, Sacchi E et al. Low platelet alpha 2 beta 1 levels of Type I von Willebrand Disease correlate with impaired platelet function in a high shear stress system. *Blood* 1999; 93: 3578–3582.
77 Santoso S, Kunicki TJ, Kroll H, Haberbosch W, Gardemann A. Association of the platelet glycoprotein Ia C_{807} T gene polymorphism with myocardial infarction in younger patients. *Blood* 1999; 93: 2449–2453.
78 Moshfegh K, Wuillemin WA, Redondo M et al. Association of two silent polymorphisms of platelet glycoprotein Ia/IIa

Cambridge Books Online © Cambridge University Press, 2009

receptor with risk of myocardial infarction: a case-control study. *Lancet* 1999; 353: 351–354.

79 Roest M, Banga JD, Grobbee DE et al. Homozygosity for 807 T polymorphism in alpha(2) subunit of platelet alpha(2)beta(1) is associated with increased risk of cardiovascular mortality in high-risk women. *Circulation* 2000; 102: 1645–1650.

80 Carlsson LE, Santoso S, Spitzer C, Kessler C, Greinacher A. The alpha 2 gene coding sequences T_{807}/A_{873} of the platelet collagen receptor integrin alpha2 beta1 might be a genetic risk factor for the development of stroke in younger patients. *Blood* 1999; 93: 3583–3586.

81 Sacchi E, Tagliabue L, Duca F, Landi G, Martinelli I, Mannucci PM. A C807T substitution in the coding sequence of the platelet collagen receptor integrin 1 as a genetic risk factor for stroke in young patients. *Thromb Haemost* 1999; Suppl. (August): 848–848.

82 Reiner AP, Kumar PN, Schwartz SM et al. Genetic variants of platelet glycoprotein receptors and risk of stroke in young women. *Stroke* 2000; 31: 1628–1633.

83 Matsubara Y, Murata M, Maruyama T et al. Association between diabetic retinopathy and genetic variations in alpha-2 beta-1 integrin, a platelet receptor for collagen. *Blood* 2000; 95: 1560–1564.

84 Corral J, Gonzalez-Conejero R, Rivera J, Ortuno F, Aparicino P, Vicente V. Role of the 807 C/T polymorphisms of the alpha2 gene in platelet GP Ia collagen receptor expression and function. *Thromb Haemostas* 1999; 81: 951–956.

85 Carlsson LE, Potzsch B, Santoso S, Greinacher A. The GPIa–C807T polymorphism on the platelet collagen receptor GPIa–IIa and the development of deep vein thrombosis. *Thromb Haemost* 1999; Suppl. (August): 254.

86 Dinauer DM, Friedman KD, Hessner MJ. Allelic distribution of the glycoprotein Ia (alpha2-integrin) C807T/G873A dimorphisms among caucasian venous thrombosis patients and six racial groups. *Br J Haematol* 1999; 107: 563–565.

87 Tarantino MD, Kunicki TJ, Nugent DJ. The estrogen receptor is present in human megakaryocytes. *Ann N Y Acad Sci* 1994; 714: 293–296.

88 Khetawat G, Faraday N, Nealen ML et al. Human megakaryocytes and platelets contain the estrogen receptor beta and androgen receptor (AR): testosterone regulates AR expression. *Blood* 2000; 95: 2289–2296.

89 Zutter MM, Mazoujian G, Santoro SA. Decreased expression of integrin adhesive protein receptors in adenocarcinoma of the breast. *Am J Pathol* 1990; 137: 863–870.

90 Moroi M, Okuma M, Jung SM. Platelet adhesion to collagen-coated wells: analysis of this complex process and a comparison with the adhesion to matrigel-coated wells. *Biochim Biophys Acta Mol Cell Res* 1992; 1137: 1–9.

91 Moroi M, Jung SM, Okuma M, Shinmyozu K. A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion. *J Clin Invest* 1989; 84: 1440.

92 Sugiyama T, Okuma M, Ushibucki F, Sensaki S, Kanaji K, Uchino H. A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia. *Blood* 1987; 69: 1712–1720.

93 Poole A, Gibbins JM, Turner M et al. The Fc receptor gamma-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *EMBO J* 1997; 16: 2333–2341.

94 Gibbins JM, Okuma M, Farndale R, Barnes M, Watson SP. Glycoprotein VI is the collagen receptor in platelets which underlies tyrosine phosphorylation of the Fc receptor gamma-chain. *FEBS Lett* 1997; 413: 255–259.

95 Tsuji M, Ezumi Y, Arai M, Takayama H. A novel association of Fc receptor gamma-chain with glycoprotein VI and their co-expression as a collagen receptor in human platelets. *J Biol Chem* 1997; 272: 23528–23531.

96 Gibbins J, Asselin J, Farndale R, Barnes M, Law CL, Watson SP. Tyrosine phosphorylation of the Fc receptor gamma-chain in collagen-stimulated platelets. *J Biol Chem* 1996; 271[30]: 18095–18099.

97 Morton LF, McCulloch IY, Barnes MJ. Platelet aggregation by a collagen-like synthetic peptide. *Thromb Res* 1993; 72: 367–372.

98 Asselin J, Gibbins JM, Achison M et al. A collagen-like peptide stimulates tyrosine phosphorylation of syk and phospholipase Cγ2 in platelets independent of the integrin alpha-2 beta-1. *Blood* 1997; 89: 1235–1242.

99 Polgar J, Clemetson JM, Kehrel BE et al. Platelet activation and signal transduction by convulxin, a C-type lectin from *Crotalus durissus terrificus* (Tropical Rattlesnake) venom via the p62/GPVI coillagen receptor. *J Biol Chem* 1997; 272: 13576–13583.

100 Jandrot-Perrus M, Lagrue AH, Okuma M, Bon C. Adhesion and activation of human platelets induced by convulxin involve glycoprotein VI and integrin alpha-2 beta-1. *J Biol Chem* 1997; 272: 27035–27041.

101 Clemetson JM, Polgar J, Magnenat E, Wells TNC, Clemetson KJ. The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcαR and the natural killer receptors. *J Biol Chem* 1999; 274: 29019–29024.

102 Fong AM, Santoro SA. Transcriptional regulation of α_{IIb} integrin gene expression during megakaryocytic differentiation of K562 cells. Role of a silencer element. *J Biol Chem* 1994; 269: 18441–18447.

103 Prandini M-H, Uzan G, Martin F, Thevenon D, Marguerie G. Characterization of a specific erythromegakaryocytic enhancer within the glycoprotein IIb promoter. *J Biol Chem* 1992; 267: 10370.

104 Martin D, Prandini ML, Thevenon D, Marguerie G, Uzan G. The transcription factor GATA-1 regulates the promoter activity of the platelet glycoprotein IIb gene. *J Biol Chem* 1993; 268: 21606–21612.

105 Block KL, Ravid K, Phung QH, Poncz M. Characterization of regulatory elements in the 5′-flanking region of the rat GPIIb gene by studies in a primary rat marrow culture system. *Blood* 1994; 84: 3385–3393.

Cambridge Books Online © Cambridge University Press, 2009

106 Block KL, Shou Y, Poncz M. An Ets/Spl interaction in the 5′-flanking region of the megakaryocyte-specific alpha IIb gene appears to stabilize Sp1 binding and is essential for expression of this TATA-less gene. *Blood* 1996; 88: 2071–2080.
107 Thornton MA, Poncz M, Korostishevsky M, Yakobson E, Usher S, Seligsohn U et al. The human platelet alpha IIb gene is not closely linked to its integrin partner beta 3. *Blood* 1999; 94: 2039–2047.
108 Gaines P, Geiger JN, Knudsen G, Seshasayee D, Wojchowski DM. GATA-1-and FOG-dependent activation of megakaryocytic alpha IIB gene expression. *J Biol Chem* 2000; 275: 34114–34121.
109 Tsang AP, Fujiwara Y, Hom DB, Orkin SH. Failure of megakaryopoiesis and arrested erythropoiesis in mice lacking the GATA-1 transcriptional cofactor FOG. *Genes Dev* 1998; 12: 1176–1188.
110 Deveauz S, Filipe A, Lemarchandel V, Ghysdael J, Romeo PH, Mignotte V. Analysis of the thrombopoietin receptor (MPL) promoter implicates GATA and Ets proteins in the coregulation of megakaryocyte-specific genes. *Blood* 1996; 87: 4678–4685.
111 Minami T, Tachibana K, Imanishi T, Doi T. Both Ets-1 and GATA-1 are essential for positive regulation of platelet factor 4 gene expression. *Eur J Biochem* 1998; 258: 879–889.
112 Ody C, Vaigot P, Quere P, Imhof BA, Corbel C. Glycoprotein IIb–IIIa is expressed in Avian multilineage hematopoietic progenitor cells. *Blood* 1999; 9: 2898–2906.
113 Block KL, Ponca M. Platelet glycoprotein IIb gene expression as a model of megakaryocyte-specific expression. *Stem Cells* 1995; 13: 135–145.
114 Marguerie G, Troesch A, Franchet P et al. Platelet GPH11B as a marker for megakaryocytopoiesis. *Prog Clin Biol Res* 1990; 356: 55–64.
115 Uzan G, Prandini MH, Berthier R. Regulation of gene transcription during the differentiation of megakaryocytes. *Thromb Haemost* 1995; 74: 210–212.
116 Albaneses P, Leboeuf M, Rosa JP, Uzan G. Identification of a GATA-overlapping sequence within the enhancer of the murine GPIIb promoter that induces transcriptional deregulation in human K562 cells. *Blood* 2000; 96: 1348–1357.
117 Noris P, Simsek S, De Bruijne-Admiraal LG et al. Max[a], a new low-frequency platelet-specific antigen localized on glycoprotein IIb, is associated with neonatal alloimmune thrombocytopenia. *Blood* 1995; 86: 1019–1026.
118 Lyman S, Aster RH, Visentin GP, Newman PJ. Polymorphism of human platelet membrane glycoprotein IIb associated with the Bak[a]/Bak[b] alloantigen system. *Blood* 1990; 75: 2343–2348.
119 Newman PJ, Derbes RS, Aster RH. The human platelet alloantigens, PLA1 and PLA2, are associated with a leucine33/proline33 amino acid polymorphism in membrane glycoprotein IIIa, and are distinguishable by DNA typing. *J Clin Invest* 1989; 83: 1778–1781.
120 Peyruchaud O, Bourre F, Morel-Kopp M-C et al. HPA-10w[b] (La[a]): Genetic determination of a new platelet-specific alloantigen on glycoprotein IIIa and its expression in COS-7 cells. *Blood* 1997; 89: 2422–2428.
121 Wang R, Furihata K, McFarland JG, Friedman K, Aster RH, Newman PJ. An amino acid polymorphism within the RGD binding domain of platelet membrane glycoprotein IIIa is responsible for the formation of the Pen[a]/Pen[b] alloantigen system. *J Clin Invest* 1992; 90: 2038–2043.
122 Kuijpers RWAM, Simsek S, Faber NM, Goldschmeding R, Van Wermerkerken RKV, Von dem Borne AEGK. Single point mutation in human glycoprotein IIIa is associated with a new platelet-specific alloantigen (Mo) involved in neonatal alloimmune thrombocytopenia. *Blood* 1993; 81: 70–76.
123 Wang R, McFarland JG, Kekomaki R, Newman PJ. Amino acid 489 is encoded by a mutational 'hot spot' on the beta 3 integrin chain: the CA/TU human platelet alloantigen system. *Blood* 1993; 82: 3386–3391.
124 Santoso S, Kalb R, Kiefel V, Mueller-Eckhardt C, Newman PJ. A point mutation leads to an unpaired cysteine residue and a molecular weight polymorphism of a functional platelet beta 3 integrin subunit. The Sra alloantigen system of GPIIIa. *J Biol Chem* 1994; 269: 8439–8444.
125 Unkelbach K, Kalb R, Breitfeld C, Santoso S, Kiefel V, Mueller-Eckhardt C. New polymorphism on platelet glycoprotein IIIa gene recognized by endonuclease *Msp* I: implications for Pl[A] typing by allele-specific restriction analysis. *Transfusion* 1994; 34: 592–595.
126 Weiss EJ, Bray PF, Tayback M et al. A polymorphism of a platelet glycoprotein receptor as an inherited risk factor for coronary thrombosis. *N Engl J Med* 1996; 334: 1090–1094.
127 Kastrati A, Schomig A, Seyfarth M et al. PIA polymorphism of platelet glycoprotein IIIa and risk of restenosis after coronary stent placement. *Circulation* 1999; 99: 1005–1010.
128 Walter DH, Schachinger V, Elsner M, Dimmeler S, Zeiher AM. Platelet glycoprotein IIIa polymorphisms and risk of coronary stent thrombosis. *Lancet* 1997; 350: 1217–1219.
129 Salido E, Martin B, Barrios Y et al. The PIA2 polymorphism of the platelet glycoprotein IIIA gene as a risk factor for acute renal allograft rejection, *J Am Soc Nephrol* 1999; 10: 2599–2605.
130 Vijayan KV, Goldschmidt-Clermont PJ, Roos C, Bray PF. The PI(A2) polymorphism of integrin beta(3) enhances outside-in signaling and adhesive functions. *J Clin Invest* 2000; 105: 793–802.
131 Michelson AD, Furman MI, Goldschmidt-Clermont P et al. Platelet GP IIIa PI(A) polymorphisms display different sensitivities to agonists. *Circulation* 2000; 101: 1013–1018.
132 Ito T, Ishida T, Shimodaira S, Kitano K. Polymorphisms of platelet membrane glycoprotein Ib alpha and plasma von Willebrand factor antigen in coronary artery disease. *Int J Hematol* 1999; 70: 47–51.
133 O'Connor F, Molloy A, Shields DC, Fitzgerald DJ. The influence of the platelet PlA2 polymorphism on the response to a glycoprotein IIb/IIIa antagonist in patients with acute coronary syndromes. *Blood* 1999; 94[10]: 244a.
134 Baker R, Lofthouse E, Staples N, Shen Y, Berndt MC, Lopez JA

Cambridge Books Online © Cambridge University Press, 2009

et al. Platelet glycoprotein (GP) Ib polymorphisms and risk of stroke. *Thromb Haemost* 1999; Suppl.(August): 2680–2680.
135 Sonoda A, Murata M, Ito D, Tanahashi N, Ohta A, Tada Y et al. Association between platelet glycoprotein Ib alpha genotype and ischemic cerebrovascular disease. *Stroke* 2000; 31: 493–497.
136 Hooper WC, Benson J, Jamil MEL, Dilley A, Wenger NK, Evatt BL. The lack of an association of the HPA-2B polymorphism of the glycoprotein Ib-alpha gene and the Glu298asp variant of the endothelial cell nitric oxide synthase gene with myocardial infarction in African–Americans. *Thromb Haemost* 1999; Suppl.(August): 189–189.
137 Tompkins K, Cameron C, Raj S, Keightley A, Lillicrap D. Evaluation of the glycoprotein Ib Kozak sequence and variable number tandem repeat polymorphisms as risk factors for unstable angina. *Thromb Haemost* 1999; Suppl. (August): 404.
138 Hillmann A, Shields D, Fitzgerald DJ, Kenny D. Polymorphisms in glycoprotein (GP) Ib alpha are not associated with adverse outcomes in primigravidae. *Thromb Haemost* 1999; Suppl.(August): 536.
139 Frank MB, Reiner AP, Arbogast PG, Schwartz M, Rosendaal FR, Siscovick DS. The Kozak sequence polymorphism of platelet glycoprotein Ib alpha and risk of myocardial infarction and stroke in young women. *Blood* 1999; 97: 875–879.
140 Corral J, Lozano ML, Gonzalez-Conejero R et al. A common polymorphism flanking the ATG initiator codon of GPIb alpha does not affect expression and is not a major risk factor for arterial thrombosis. *Thromb Haemost* 2000; 83: 23–28.
141 Corral J, Gonzalez-Conejero R, Iniesta JA, Martinez C, Lozano ML, Rivera J et al. New GPIb polymorphism: Role in thromboembolic disease. *Thromb Haemost* 1999; Suppl.(August): 539.
142 Croft SA, Hampton KK, Sorrell JA, Steeds RP, Channer KS, Samani NJ et al. The GPIa C807T dimorphism associated with platelet collagen receptor density is not a risk factor for myocardial infarction. *Br J Haematol* 1999; 106: 771–776.
143 Matsubara Y, Murata M, Maruyama T, Handa M, Saruta T, Ikeda Y. A polymorphism in a platelet collagen receptor (21 integrin) is associated with diabetic retinopathy. *Thromb Haemost* 1999; Suppl.(August): 360.
144 Hessner MJ, Dinauer DM, Luhm RA, Endres JL, Montgomery RR, Friedman KD. Contribution of the glycoprotein Ia 807TT, methylene tetrahydrofolate reductase 677TT and prothrombin 20210GA genotypes to prothrombotic risk among factor V 1691 GA (Leiden) carriers. *Br J Haematol* 1999; 106: 237–239.
145 Carter AM, Ossei-Gerning N, Grant PJ. Platelet glycoprotein IIIa PIA polymorphism in young men with myocardial infarction. *Lancet* 1996; 348: 485–486.
146 Zotz RB, Winkelmann BR, Nauck M et al. Polymorphism of platelet membrane glycoprotein IIIa: human platelet antigen 1b (HPA-1b/PIA2) is an inherited risk factor for premature myocardial infarction in coronary artery disease. *Thromb Haemost* 1998; 79: 731–735.
147 Anderson JL, King GJ, Blair TL et al. Associations between a polymorphism in the gene encoding glycoprotein IIIa and myocardial infarction or coronary artery disease *J Am Coll Cardiol* 1999; 33: 727–733.
148 Mikkelsson J, Perola M, Laippala P et al. Glycoprotein IIIa PI(A) polymorphism associates with progression of coronary artery disease and with myocardial infarction in an autopsy series of middle-aged men who died suddenly. *Arterioscler Thromb Vasc Biol* 1999; 19: 2573–2578.
149 Tereschchenko SN, Levchuk NN, Drozdov VN et al. Polymorphism of GPIIIA platelet glycoprotein gene PIA1/A2 compared to plasma hemostasis in myocardial infarction patients. *Ter Arkh* 1999; 71: 66–70.
150 Ardissino D, Mannucci PM, Merlini PA et al. Prothrombotic genetic risk factors in young survivors of myocardial infarction. *Blood* 1999; 94: 46–51.
151 Mikkelsson J, Perola M, Laippala P, Penttila A, Karhunen PJ. Glycoprotein IIIa Pl(A1/A2) polymorphism and sudden cardiac death. *J Am Coll Cardiol* 2000; 36: 1317–1323.
152 Yu Q, Safavi F, Roberts R, Marian AJ. A variant of beta fibrinogen is a genetic risk factor for coronary artery disease and myocardial infarction. *J Investig Med* 1996; 44: 154–159.
153 Osborn SV, Hampton KK, Smillie D, Channer KS, Daly ME. *Lancet* 1996; 348: 1309–1310.
154 Herrmann SM, Poirier O, Marques-Vidal P et al. The Leu33/Pro polymorphism (Pl^{A1}/Pl^{A2}) of the glycoprotein IIIA (GPIIIa) receptor is not related to myocardial infarction in the ECTIM study. *Thromb Haemost* 1997; 77: 1179–1181.
155 Ridker PM, Hennekens CH, Schmitz C, Stampfer MJ, Lindpaintner K. $Pl^{A1/A2}$ polymorphism of platelet glycoprotein IIIa and risks of myocardial infarction, stroke, and venous thrombosis. *Lancet* 1997; 349: 385–388.
156 Samani NJ, Lodwick D. Glycoprotein IIIa polymorphism and risk of myocardial infarction. *Cardiovasc Res* 1997; 33: 693–697.
157 Durante-Mangoni E, Davies GJ, Ahmed N, Ruggiero G, Tuddenham EG. Coronary thrombosis and the platelet glycoprotein IIIA gene PLA^2 polymorphism. *Thromb Haemost* 1998; 80: 218–219.
158 Gardemann A, Humme J, Stricker J et al. Association of the platelet glycoprotein IIIa $Pl^{A1/A2}$ gene polymorphism to coronary artery disease but not to nonfatal myocardial infarction in low risk patients. *Thromb Haemost* 1998; 80: 214–217.
159 Mamotte CD, van Bockxmeer FM, Taylor RR. Pla1/a2 polymorphism of glycoprotein IIIa and risk of coronary artery disease and restenosis following coronary angioplasty. *Am J Cardiol* 1998; 82: 13–16.
160 Scaglione L, Bergerone S, Gaschino G et al. Lack of relationship between the P1A1/P1A2 polymorphism of platelet glycoprotein IIIa and premature myocardial infarction. *Eur J Clin Invest* 1998; 28: 385–388.
161 Moshfegh K, Wuillemin WA, Redondo M, Lammle B, Liechti S, Meyer BJ. Platelet polymorphisms of GPIa/IIa (Collagen) and GPIIb/IIIa (Fibrinogen) receptors and the risk of myocardial infarction. *Thromb Haemost* 1999; Suppl.(August): 361.
162 Kekomaki S, Hamalainen L, Kauppinen-Makelin R, Palomaki

Cambridge Books Online © Cambridge University Press, 2009

H, Kaste M, Kontula K. Genetic polymorphism of platelet glycoprotein IIIa in patients with acute myocardial infarction and acute ischaemic stroke. *J Cardiovasc Risk* 1999; 6: 13–17.
163 Bottiger C, Kastrati A, Koch W et al. HPA-1 and HPA-3 polymorphisms of the platelet fibrinogen receptor and coronary artery disease and myocardial infarction *Thromb Haemost* 2000; 83: 559–562.
164 Corral J, Gonzalez-Conejero R, Rivera J, Iniesta JA, Lozano ML, Vicente V. HPA-1 genotype in arterial thrombosis – role of HPA-1b polymorphism in platelet function. *Blood Coag Fibrinol* 1997; 8: 284–290.
165 Garcia-Ribes M, Gonzalez-Lamuno D, Hernandez-Estefania R et al. Polymorphism of the platelet glycoprotein IIIa gene in patients with coronary stenosis. *Thromb Haemost* 1998; 79: 1126–1129.
166 Laule M, Cascorbi I, Stangl V et al. A1/A2 polymorphism of glycoprotein IIIa and association with excess procedural risk for coronary catheter interventions: a case-controlled study. *Lancet* 1999; 353: 708–712.
167 Kastrati A, Koch W, Gawaz M et al. PlA polymorphism of glycoprotein IIIa and risk of adverse events after coronary stent placement. *J Am Coll Cardiol* 2000; 36: 84–89.
168 Wagner KR, Giles WH, Johnson CJ et al. Platelet glycoprotein receptor IIIa polymorphism P1A2 and ischemic stroke risk: The Stroke Prevention in Young Women Study. *Stroke* 1998; 29: 581–585.
169 Carter AM, Catto AJ, Bamford JM, Grant PJ. Platelet GP IIIa PlA and GP Ib variable number tandem repeat polymorphisms and markers of platelet activation in acute stroke. *Arterioscler Thromb Vasc Biol* 1998; 18: 1124–1131.
170 Croft SA, Hampton KK, Daly ME, Steeds RP, Channer KS, Samani NJ. Kozak sequence polymorphism in the platelet GPIb alpha gene is not associated with risk of myocardial infarcation. *Blood* 2000; 95: 2183–2184.

Cambridge Books Online © Cambridge University Press, 2009

Cambridge Books Online © Cambridge University Press, 2009

Part III

C Non-hemostatic disorders

Cambridge Books Online © Cambridge University Press, 2009

Cambridge Books Online © Cambridge University Press, 2009

51

Platelets and bacterial infections

Mark C. Herzberg and Ke Gong

Department of Preventive Sciences, School of Dentistry, University of Minnesota, USA

Introduction

Platelet–bacterial interactions contribute to the pathogenesis of hematological infections. Interactions may occur directly with platelets, resulting in bacterial invasion or induction of platelet aggregation. Bacterial invasion of platelets is a key feature of Rickettsial and Ehrlichial infections. Induction of platelet aggregation in vivo, resulting in formation of mural thrombi on heart valves in infective endocarditis, appears to be a response to certain infecting bacteria. During bacteremia or sepsis, platelet microaggregates form and thrombocytopenia is observed. The propensity for bacterial cells to trigger platelet aggregation and thrombosis in vivo suggests concomitant procoagulant activation. Procoagulant activity can also be up-regulated by bacterial products, such as lipopolysaccharides (LPS) and expression of thrombin-like enzymes, resulting in the incorporation of platelets into aggregates or thrombi. Conversely, certain bacteria express toxins and other proteases that will inhibit platelet function or cause platelet lysis and contribute to coagulopathy. More than 30 species of bacteria show platelet activating or inhibitory functions, many of which have been documented to occur during naturally occuring infection in mammals or in experimental animal or human models. This chapter will present examples of platelet dysfunction that results from clinical infections, followed by an examination of partnered bacterial ligands and platelet receptor or targets, and will conclude with a survey of key mechanistic models of platelet–bacterial interactions.

That bacteria in the blood interact with circulating platelets has been recognized since early in the last century (see review by Clawson)[1]. Platelet–bacterial interactions were considered by many investigators to be an important feature of reticuloendothelial defense, although the supporting data were often not widely appreciated. At the turn of the last century, rabbits were shown to clear experimental bacteremias with *Vibrio cholera* by clumping with platelets and several groups reported similar observations during the next twenty years. In 1922, a report published by Bull and McKee showed that thrombocytopenic and control rabbits cleared Staphylococcal bacteremias similarly. Earlier reports notwithstanding, investigators shifted focus away from the platelet as interactive with bacteria in the blood. In 1930, interest resurfaced as Taniguchi and coworkers reported that experimental bacteremias with selected Gram-positive or Gram-negative species caused thrombocytopenia within 3 minutes, formation of platelet–bacterial aggregates, and disappearance of free bacteria by 10 minutes. The numbers of platelet-associated bacteria far exceeded those associated with leukocytes. Platelets reappeared in the circulation by 30 minutes. Direct observation of circulating platelet–bacteria aggregates in vivo waited until the 1960s, when Alfred Copley and colleagues developed a vital microscopic approach. While platelet–neutrophil interactions, described by Aaron Marcus and coworkers in the 1980s, could be modified by circulating bacteria and contribute to reticuloendothelial clearance, we are still coming to appreciate the potential for platelet–bacterial interactions to contribute to health and disease.

Platelet diseases in humans or animals caused by bacteria

Bacteria directly invading platelets – Ehrlichial and Rickettsial infections

Rickettsial and Ehrlichial infections are often associated with direct bacterial invasion of platelets. The Rickettsieae and Ehrlichieae are Gram-negative intracellular bacterial

pathogens. The human ehrlichioses and Rocky Mountain spotted fever are life-threatening yet treatable diseases. The vectors for these bacteria are hard-bodied ixodid ticks, and the reservoir hosts are probably wild rodents, deer, sheep and dogs. Prompt treatment with tetracycline or chloramphenicol markedly decreases the morbidity[2], yet the differential diagnoses may prove difficult to ascertain when treatment decisions are needed. Each presents as a non-specific febrile illness that can be followed by immunosuppression leading to secondary infection. Most patients first encounter fever, headache, chills, and myalgias and then develop anemia, elevations in serum hepatic aminotransferases, and thrombocytopenia, with or without leukopenia[2–5]. When patients are seropositive to Ehrlichiae by an indirect fluorescent antibody test (IFAT)[6,7], clusters of bacteria can frequently be found in the cytoplasm of peripheral blood neutrophils[8]. Sometimes, Ehrlichiae are not detected in peripheral blood smears, there are no antibodies to these diverse agents at the time of presentation, and isolation does not yield sensitive and timely results. For diagnosis, polymerase chain reaction (broad-range and species-specific PCR) assay promises the greatest sensitivity, specificity and timeliness[9–11].

Both diseases are tick-borne and the effects on blood cells can be similar in humans and canines[12]. The Rickettsia include the spotted fever group (Rocky Mountain or Mediterranean spotted fevers) and the bacterium primarily infects endothelial cells[13]. The Ehrlichieae species that infect humans include *E. chaffeensis, E. sennetsu, E. canis, E. ewingii, and E. platys*[10,12,14–16]. The Ehrlichieae are remarkable for tropism to neutrophils[4,14]. About 80% of serum samples from confirmed human granulocytic ehrlichiosis patients contain antiplatelet antibodies, which may contribute to profound thrombocytopenia[17]. Experimental Rickettsia and Ehrlichia infections in dogs also show elevated platelet-associated immunoglobulin[18]. Rickettsia and ehrlichia may interact with platelets through cell surface receptor(s) common also to endothelial cells and neutrophils.

The emergence of antiplatelet antibodies and activation of complement[17,18] appears to follow morphological, functional changes and destruction of platelets[19,20]. In naturally occurring and experimental canine ehrlichiosis, for example, platelets are rounded, show reduced pseudopod formation, clumping, migration and leakage, and these features are followed by pancytopenia[19]. In vitro, platelet responses to agonists are also inhibited. In response to collagen/epinephrine, thrombin and adenosine diphosphate (ADP), aggregation is attenuated with an increase in the lag time to onset, and a decrease in the maximal extent and rate[20,21]. Similarly, platelet factor 3 (PF-3) release is delayed[22]. The bleeding tendency and thrombocytopenia in acute phase ehrlichial/rickettsial infections, therefore, appear to be caused by direct effects of the bacteria on platelets, and emergence of specific autoantibodies.

Bacteria promoting platelet aggregation and coagulation: infective endocarditis

Infective endocarditis describes a family of persistent microbial infections that typically target previously damaged or diseased heart valves. Most often, infective endocarditis occurs after bacteremia containing viridans streptococci or *Staphylococcus aureus* although the specific breaching event into the blood may be uncertain[23–25]. The viridans streptococci, commensal Gram-positive bacteria, are of particular interest in infective endocarditis. They are harmless on the mucous membranes, but behave as pathogens in the blood. Oral and dental infections appear to be the most common portals of entry for infecting bacteria. For *Staphylococcus aureus*-associated endocarditis, the risk is often associated with intravenous drug abuse. The infecting microbes colonize within a thrombus-like mass of platelets and fibrin.

The platelet-fibrin 'vegetations' sequester and protect the microbial colonies from both the innate and adaptive immune systems and from therapeutic intervention with antibiotics. The ability of the bacteria to interact with platelets and promote their aggregation in vitro and in vivo appears to be linked directly to the mass of the vegetation and the severity of the clinical signs[25–27]. The ability of strains to induce platelet aggregation generally depends on their ability to adhere to platelets[28–30] and to bind to platelet receptors[31,32]. Consistent with these observations, the frequency of infection in experimental rabbit endocarditis was associated with the ability of *Staphylococcus aureus* to bind to platelets[33] and/or fibrin–platelet–endothelium matrix[34]. Potentially important virulence factors in the pathogenesis of endocarditis include lipoteichoic acid (LTA) of group A streptococci and *Staphylococcus aureus*[34–37], platelet aggregation-associated protein (PAAP) of *Streptococcus sanguis*[26], streptococcal exopolysaccharides[38] and resistance to platelet microbicidal proteins[39].

Usually infective endocarditis presents as non-specific symptoms including malaise, fever, sweats, myalgia, weight loss, and sustained elevations of C-reactive protein, erythrocyte sedimentation rate, and other inflammatory markers[40–42]. Cardiac murmur, tachycardia, vascular phenomena and a change in mental state are also commonly noted. Active disease is characterized by dynamic changes in coagulation and platelet aggregation, the increase in acute-phase reactants[43], and a tendency towards compli-

Cambridge Books Online © Cambridge University Press, 2009

cating thrombotic events[44]. Patients also show elevated antiphospholipid antibodies associated with endothelial cell activation, thrombin generation and impairment of fibrinolysis[45].

Bacterial strains that activate coagulation would be expected to trigger formation of larger vegetations, causing disease with a more complicated clinical course. Even though they are considered to be commensals with little pathogenic potential, viridans streptococci activate procoagulant and thrombotic mechanisms. The viridans streptococci express thrombin- and Hageman factor-like enzymes[46], promote expression of tissue factor by endothelial cells[47] or monocytes[48,49], and induce platelet aggregation[30,50–52]. While Bernard–Soulier platelets, lacking platelet glycoprotein Ib/V/IX, aggregate in response to *S. sanguis*, and GPIa and GPIIb/IIIa are necessary[32], the ability to engage a full coagulant response is required. For example, platelets from experimental pigs with von Willebrand disease and normal pigs aggregate in response to group C streptococci[53]. Yet the von Willebrand pigs fail to develop experimental endocarditis in response to the group C streptococci[53]. von Willebrand factor (VWF) binds platelet glycoprotein Ib/V/IX, which is not crucial to streptococcal-induced platelet aggregation. Since VWF protects cocirculating factor VIII against degradation, the data suggest that valvular thrombus formation in response to group C streptococcus infection requires activation of coagulation through factor VIII[53]. The procoagulant effect of the bacteria during infection is crucial to the development of the vegetation. Platelet aggregation at the heart valve is necessary, but not sufficient, since specific antibody blockade of the *S. sanguis* PAAP (see above) reduces, but does not eliminate the thrombotic vegetation in experimental rabbits[54].

After infection, strains releasing proteases and other tissue hydrolases can cause necrosis of the vegetations and, over time, valvular destruction and insufficiency[55]. For example, proteolytic strains of *Enterococcus faecalis* cause partial dissolution of experimental vegetations in rabbits, resulting in high levels of bacteremia, small, soft, friable vegetations, a high frequency of kidney infarcts, and shorter survival times[56]. As a result, thromboembolic events and severe complications of infective endocarditis are common. The characteristics of the embolic events are also related to the nature of the pathogen.

Antibiotic therapy is used to control the infection. Infecting microbes are more inaccessible to systemic antibiotics when embedded within the dense platelet–fibrin matrix of the vegetations than when they are colonizing the periphery of the vegetation. Platelets however express the platelet microbicial proteins (PMPs), which limit the colonization of sensitive bacteria and may act synergistically with certain antibiotics[39].

Disseminated intravascular coagulation (DIC)

DIC is an acquired syndrome characterized by intravascular fibrin formation, and depletion of platelets and coagulation factors in the course of a variety of severe diseases, most commonly infectious septicemia[57]. Intravascular fibrin contributes to thrombotic occlusion of blood vessels, compromising the blood supply to organs, while ongoing coagulation, diminished availability of platelets and depletion of coagulation factors contribute to episodes of bleeding. In DIC, platelets may be activated by direct interactions with certain bacteria[27,58]. With the generalized activation of coagulation, bacteria also activate platelets indirectly. Bacteria or their products, such as lipopolysaccharides (LPS), activate tissue factor and factor VIIa, generating thrombin. Thrombin is a potent platelet agonist and activates procoagulant activity on the platelet surface[59,60], and also promotes formation of systemic fibrin deposits. Thrombosis of mid- and small-sized vessels results in organ failure[61–63]. In DIC, suppression of anticoagulation and the delayed removal of fibrin may also contribute to the extent of coagulation.

In the presence of thrombocytopenia and depleted coagulation factors, DIC can present as a bleeding and a thrombotic disorder. In view of this seemingly contradictory presentation, DIC presents diagnostic and therapeutic challenges. DIC is diagnosed with supporting laboratory analysis of fibrinogen/fibrin degradation products (FDP; F1 + 2), D-dimer concentration, antithrombin III (AT III), thrombin–antithrombin complex (TAT), plasmin–plasmin inhibitor complex (PPIC), soluble fibrin monomer (sFM), partial thromboplastin time, prothrombin time and platelet counts[64–68]. In patients with DIC, activated platelets release thrombopoietin and the elevated concentration correlates with markers for thrombin generation, TAT complexes and F1 + 2[69]. Bacterial enzymes such as streptokinase and staphylokinase promote fibrinolysis as mimics of plasminogen activator[70]. Expression of laboratory markers of coagulation and platelet status can result from direct actions of bacteria or their products.

Bacterial enzymes can directly activate coagulation[71] and, depending on environmental conditions, also express enzymes that dissolve clots[72]. An extracellular cysteine proteinase of *Streptococcus pyogenes* can directly hydrolyse fibrinogen[73]. Two arginine-specific cysteine proteinases of *Porphyromas gingivalis* can activate and consume protein C, contributing potentially to a thrombotic tendency[74]. While bacterial products can modulate the formation and

Cambridge Books Online © Cambridge University Press, 2009

lysis of clots, direct bacterial interactions with platelets can initiate a procoagulant effect and release of thrombopoietin and other markers of DIC.

Bacteria may even more directly contribute to the consumption of coagulation factors and promote the generation of proinflammatory kinins[75]. Protein–protein interactions on the surfaces of certain Gram-negative and, perhaps, Gram-positive bacteria result in the assembly of factor XI, XII, plasma prokallikrein and high molecular weight kininogen, resulting in activation of the contact-phase system and release of bradykinin from plasma[76]. Generation of bradykinin at the bacterial surface was accompanied by prolonged clotting time, altered formation of fibrin clots, and a hypocoagulatory state in vivo. During infections, certain bacteria appear to be able to directly consume coagulation factors, independent of the activation of tissue factor. Release of kinins would contribute to the inflammatory signs of sepsis and the bleeding and epitaxis common to DIC, and by blocking the intrinsic pathway of coagulation promote hematogenous spread of the bacteria.

Hence it is not surprising that Gram-positive and Gram-negative bacteria are associated with DIC with similar frequencies[57], and clinical and laboratory presentations[77]. Diffuse coagulation is activated by the Gram-positive *Staphylococcus aureus*[78,79], *Streptococcus sanguis*[80–82], and *Streptococcus pneumoniae*[83]. The Gram-negative pathogens considered most often to be etiological are *Escherichia coli*[84] and *Neisseria meningitidis*[65,83,85]. In the United States, for example, *N. meningitidis* is the leading cause of bacterial meningitis and meningococcal sepsis in children and young adults[85]. Despite the availability of potent antibiotics, mortality in meningococcal disease remains high (about 10%), rising to 40% of patients presenting with severe shock and concurrent DIC[65]. In addition to eliciting procoagulant effects, most of these species will induce platelet aggregation in vitro in a strain-specific manner, suggesting that direct interactions with platelets can occur in vivo. In Gram-negative sepsis, endotoxin is suggested to elicit tissue factor-dependent hypercoagulation, mediated at least in part by proinflammatory upregulation of cytokines such as tumour necrosis factor-alpha and interleukin-6[86].

Thrombocytopenia frequently occurs in DIC, generally in association with hypercoagulation. Thrombocytopenia is characterized by procoagulant microparticles typically formed by aggregated, activated platelets[87,88], although reduced thrombopoiesis and immunological platelet damage may contribute[62,63]. The formation of circulating platelet microaggregates can occur in the presence of bacterial infections in the absence of sepsis and DIC. Patients with gastrointestinal *Helicobacter pylori* infections and a murine model, for example, show platelet activation and P-selectin-dependent aggregation[89]. The accompanying microvascular dysfunction and inflammatory cell infiltrate suggest that greater hematogenous spread of the *Helicobacter* or its products could cause DIC.

During DIC, tissue factor activity, which may be up-regulated directly by LPS and other bacterial products, is predominantly antagonized by plasminogen activator inhibitor-1 (PAI-1)[63,90,91]. Other natural inhibitory mechanisms of coagulation include antithrombin, the protein C system, and tissue factor pathway inhibitor. Many bacteria can enzymatically degrade selected proteins that inhibit generation of fibrin and activation of fibrinolysis. Indirectly, certain bacteria would promote decompensated DIC and thrombosis, with rapid consumption of inhibitory factors including antithrombin III (AT III) and proteins C and S[63,86]. In the clinical setting, it remains unclear if bacteria or their products actually antagonize the reversal of coagulation and thrombosis.

Bacteria may initiate or activate the coagulation cascade, and promote thrombosis by direct or indirect interactions with platelets. Circulating platelet aggregates are clinically important procoagulant particles contributing to thrombotic occlusion of microvessels and infarction and failure of major organs. Yet, when it is to their advantage, bacteria can almost paradoxically dissolve clots and consume coagulation factors, or antagonize anticoagulation mechanisms. Bacteria can promote release of kinins, activation of thrombin[92] and up-regulation of proinflammatory cytokines, creating complex molecular links between inflammation and coagulation. Confoundingly, inflammation promotes coagulation and thrombosis by promoting expression of intravascular tissue factor and leukocyte adhesion molecules, and down-regulating the fibrinolytic and protein C anticoagulant pathways. Direct and indirect bacterial interactions with platelets, therefore, may either promote or inhibit or reverse thrombosis or coagulation in DIC. Diagnostic tests must be interpreted with clear understanding of the confounding contributions of the infecting bacteria. Likewise, therapy must include antibiotic and selective anti-inflammatory treatment, but the underlying hemostatic and thrombotic disorders must be deciphered and supported with replacement therapies.

Cambridge Books Online © Cambridge University Press, 2009

Mechanisms of bacterial–platelet interactions (in vitro evidence of biological plausibility)

Bacterial ligands

Lipoteichoic acid (LTA)

The LTA of group A streptococcal and *Staphylococcus aureus* binds to platelets in a concentration- and time-dependent manner[35–37]. By immuno-ferritin labelling of LTA, binding sites on the platelet surface show a patchy distribution. In the presence of soluble LTA, collagen- and alpha1 chain-induced platelet aggregation was inhibited, but the release reaction was apparently unaffected[35]. The LTA binds to platelets in a two-site interaction, one that interferes with collagen-induced aggregation and a second that triggers the release reaction[35]. In collagen-stimulated human platelets, the LTA of *S. aureus* causes dose-dependent reduction in membrane fluidity, inhibition of phosphoinositide hydrolysis, thromboxane A2 formation, intracellular Ca^{+2} mobilization, and phosphorylation of P47, a 47 kDa marker of protein kinase C activation[36]. These results suggest that the antiplatelet activity of LTA may involve conformational changes in the platelet membrane, leading to inhibition of phospholipase C. Downstream effects would result in inhibition of phosphoinositide metabolism and thromboxane A2 formation, intracellular Ca^{+2} mobilization and protein kinase C. LTA-mediated antagonism of platelet function may, therefore, contribute to bleeding diathesis in Gram-positive septicemic patients.

The platelet aggregation-associated protein (PAAP)

Streptococcus sanguis PAAP triggers the accumulation of platelets into the valvular vegetation in experimental endocarditis[54,93]. PAAP is synthesized as a rhamnose-rich glycoprotein of 115 kDa and contains a collagen-like platelet-interactive domain, pro–gly–glu–gln–gly–pro–lys[94–96]. This platelet-interactive protein is probably homologous to the collagen-like product of the *scl* gene in group A streptococci, which promotes adhesion to epithelial cells and contributes to soft tissue pathology in a murine model[97].

Expressed on the cell wall of platelet aggregation-inducing strains (Agg+) of *S. sanguis*, PAAP apparently interacts with a signal-transducing receptor complex on platelets, which includes CD31, CD36 and CD26 and a 65-kDa collagen-binding protein (K. Gong et al., 2000, unpublished data). In experimental endocarditis in rabbits[26], PAAP first promotes platelet accumulation into a fibrin-enmeshed thrombus (vegetation), within which *S. sanguis* colonizes. When PAAP is unexpressed or neutralized with specific antibodies, experimental endocarditis runs a milder course and vegetations are smaller. From in vitro experiments, the aggregation of platelets into the vegetative thrombus may be potentiated by an ectoATPase expressed on the surface of the *S. sanguis* and platelet alpha-adrenoreceptors that respond to endogenous catecholamines. PAAP expression may be environmentally regulated during infection in response to heat shock (fever) or collagen (exposed on damaged heart valves), enabling the bacteria to more effectively recruit platelets[98]. During infection of the heart valve, at least 13 environmentally regulated virulence genes have been identified that are unexpressed in vitro[99]. The data suggest strongly, therefore, that PAAP stimulates the development of the characteristic septic mural thrombus (vegetation) of infective endocarditis and the signs of valvular pathology.

Protein A

Expressed on the surface of *Staphylococcus aureus*, protein A is a 42 kDa protein with five homologous Ig-binding domains[100]. The designated domains bind either Fcγ or Fab portions of the antibody molecule. The domains differentially recognize membrane anchored immunoglobulins defining subsets of T and B cells[100] and platelets[101]. Strains of *S. aureus* that express protein A and purified protein A bind whole platelets through gC1qR/p33, a multifunctional, ubiquitously distributed cell surface protein. Since protein A-positive *S. aureus* induce platelet aggregation[102], binding to platelet surface gC1qR/p33 may bring other ligands on the cell wall into proximity of signal transducing receptors to trigger up-regulation of glycoprotein IIb/IIIa. While the evidence does not yet support a direct role of protein A in inducing platelet aggregation, binding to activated platelets suggests an additional mechanism to localize *S. aureus* at sites of vascular injury and thrombosis.

Phospholipase C (PLC)

Strains of *S. aureus* expressing PLC are more frequently associated with adult respiratory distress syndrome and DIC than toxic shock syndrome[103]. Purified PLC irreversibly aggregates platelets after a time lag, the length of which is inversely related to the specific activity of the enzyme[104]. A substrate for PLC, *p*-nitrophenolphosphorylcholine, inhibits PLC-induced aggregation when preincubated with platelets before addition of PLC. Conversely, PLC did not inhibit aggregation in response to ADP, epinephrine, collagen or ristocetin[105]. In ex vivo experiments, oral aspirin (500 mg) partially inhibited platelet aggregation induced by PLC[106].

An extracellular lipolytic enzyme, PLC is highly con-

Cambridge Books Online © Cambridge University Press, 2009

served in prokaryotes including *Staphylococci*, *Streptococci*, *Bacillus*, *Pseudomonas*, and *Clostridium*[107–109]. This enzyme cleaves glycosylphosphatidylinositol-anchored proteins from the plasma membranes of eukaryotic cells[110]. The N-terminal domain of PLC catalyses phospholipid hydrolysis, and its C-terminal region mediates interactions with membrane phospholipids in a calcium-dependent manner[111,112]. After incubation with PLC, human platelets lose 20–45% of total phospholipid, including 50–75% of the phosphatidylethanolamine, 20–50% of phosphatidylcholine, and 20–25% of phosphatidylserine[113]. The PLC and alpha-toxin of *S. aureus* may effect similar changes in platelets. The alpha-toxin intercalates as a pore-forming hexamer in liposomes[114] and platelet plasma membranes[108], activating endogenous PLC activity and downstream Ca^{2+}-signalling in pheochromocytoma (PC12) cells[115]. In platelets, activation of these signalling pathways may trigger secretion and aggregation.

Lipopolysaccharide (LPS)

A frequently studied prototype of macromolecules found exclusively in Gram-negative bacteria LPS of *E. coli* inhibits aggregation of collagen-stimulated human platelets[116]. Like LTA of Gram-positive bacteria, LPS causes dose-dependent inhibition of phosphoinositide hydrolysis, intracellular Ca^{2+} mobilization, membrane fluidity, and protein kinase C activation. Since LPS also increased formation of cGMP and cell-associated nitrate, the antiplatelet activity of LPS may be caused by conformational changes in the platelet membrane, leading to decreased activity of phospholipase C and formation of nitric oxide (NO)/cyclic GMP. By down-regulating platelets, LPS may contribute to bleeding diathesis in septicemic and endotoxemic patients. Lipid A is the toxic moiety of LPS, causing rapid time and concentration dependent phosphorylation of a human platelet protein of M_r 47 000, a marker of protein kinase C activation, secretion of [^{14}C]serotonin, and platelet aggregation[117]. These data suggest that lipid A may represent a significant mechanism underlying broadly the hematologic and circulatory disorders observed in endotoxic shock, since protein kinase C is expressed ubiquitously in blood cells, vascular cells and neurons.

Platelet receptors

Platelet gC1qR

A promiscuous receptor of 33 kDa on human platelets and other cells, gC1qR/p33 binds *S. aureus* protein A[101] and the globular head domains on proteins including C1q, vitronectin, and high molecular weight kininogen[118]. A similar platelet protein cC1qR is 60 kDa and binds collagen-like domains on many extracelluar matrix proteins[119]. While it is unclear if *S. aureus* mediates signal through gC1qR[101], C1q multimers promote phosphoinositide hydrolysis, upregulate glycoprotein IIb/IIIa complexes, increase binding of fibrinogen and induce platelet aggregation through cC1qR[120] and gC1qR[121]. C1q multimers also promote a procoagulant effect associated with platelets. Yet expression of gC1qR/p33 is poor on resting and activated platelets, but is up-regulated after platelets bind to immobilized fibrinogen, collagen or fibronectin[121]. gC1qR binds the D-domain of fibrinogen, impairing the polymerization to fibrin[122]. It is unclear how these several observations may mediate coagulation in sites of immune injury or inflammation, but gC1qR may function to localize *S. aureus* to activated platelets at sites of vascular injury and promote infection of thrombi.

GP IIb/IIIa

Blockade of glycoprotein IIb/IIIa protects against microvascular thrombosis in response to infusion of *Escherichia coli* in a baboon model[123]. To aggregate in response to virtually all bacteria studied, platelets must bind fibrinogen/fibrin. Fibrinogen/fibrin bridges platelets to one another to form aggregates and also bridge certain bacteria such as *S. aureus* to platelets. While it is well accepted that fibrinogen/fibrin bridges between platelets requires platelets to up-regulate glycoprotein IIb/IIIa, it is less clear that this integrin receptor serves to bridge bacteria to platelets.

For example, glycoprotein IIb/IIIa does not apparently bind *S. aureus* to platelets[102,124] favouring gC1qR[101]. Glycoprotein IIb/IIIa may be required for fibrinogen-mediated platelet–platelet aggregation triggered by *Lactobacillus* species[125] and *Streptococcus sanguis*[32,93,97]. *Lactobacillus rhamnosus* induced aggregation was inhibited by the glycoprotein antagonist peptide arginine–glycine–aspartic acid–serine (RGDS)[125]. *S. sanguis* does not induce aggregation of platelets from patients with Glanzmann's thrombasthenia, which lack glycoprotein IIb/IIIa[32] and aggregation of a type strain ATCC 7863 is inhibited by monoclonal antibody to GPIIb/IIIa, RGDS peptide, and a specific antagonist for the platelet fibrinogen receptor, GPIIb/IIIa[126]. Yet it is unclear if glycoprotein IIb/IIIa directly serves as a platelet receptor for *Staphylococcus aureus*, *Lactobacilli* or *S. sanguis*.

Glycoprotein IIb/IIIa may serve as a binding site for certain group A streptococci and *Borrelia burgdorferi*. Group A streptococci express an extracellular cysteine protease (streptococcal pyrogenic exotoxin B) that is critical for tissue invasion[127]. One variant contains an RGD

Cambridge Books Online © Cambridge University Press, 2009

sequence that confers binding to glycoprotein IIb/IIIa. This subtle molecular variation may alter virulence in group A streptococcal infections and present a clinical picture reflecting altered host–pathogen interactions. Similarly infectious strains of *Borrelia burgdorferi* bind to activated platelets, in which glycoprotein IIb/IIIa is upregulated[128]. Binding is specific for activated glycoprotein IIb/IIIa since *B. burgdorferi* bound to platelets in the presence of antibodies against other receptors but failed to bind to platelets in the presence of RGD competitors or when platelets were deficient in this integrin.

GPIb

Streptococcus sanguis strain 7863 induces aspirin-sensitive platelet aggregation suggested to require glycoprotein IIb/IIIa and also glycoprotein Ib[126,129]. When glycoprotein Ib was blocked with a monoclonal antibody, platelet aggregation was inhibited. Conversely, von Willebrand factor (vWF) binding to platelets via glycoprotein Ib was not affected, nor did *S. sanguis* prevent ristocetin-induced platelet agglutination or vWF binding. Normal subjects and patients with von Willebrand's disease showed similar platelet aggregation in response to strain 7863. Other strains of *S. sanguis* induce aggregation similarly in patients with Bernard–Soulier, which lack glycoprotein Ib, and normal platelets[32]. Collectively the evidence does not support a role of glycoprotein Ib in platelet aggregation in response to *S. sanguis*.

Glycoprotein Ib is a target for cleavage by the *o*-sialoglycoprotein endoprotease of *Pasteurella hemolytica*[130]. After hydrolysis the platelets appear to function like platelets from patients with Bernard–Soulier syndrome. The platelets respond normally to most physiological agonists, including thrombin, ADP, collagen and thromboxane A2, but fail to agglutinate in response to ristocetin/vWF.

Fc gamma receptor/glycoprotein VI

Monoclonal antibody to platelet Fc receptor (FcγRII) significantly reduced *S. aureus*–platelet binding[131]. A similar monoclonal antibody against FcγRII blocks *S. sanguis* strain 7863-induced platelet aggregation[126]. Since platelet-aggregating strains of *S. sanguis* express PAAP with its collagen-like platelet-interactive motif[95,96], interactions with FcγRII might be expected. As part of glycoprotein VI, a major receptor for collagen on platelets[132], FcγRII is crucial to signal transduction and the aggregation response[133,134].

CD26, PECAM (CD31), and glycoprotein IV (CD36)

Human platelets express binding sites for *Streptococcus sanguis* that react with murine anti-idiotype monoclonal antibodies (Mab2s) (raised against murine Mab anti-*S. sanguis* adhesins)[31]. To identify the target antigens, the Mab2.2 was used to immunoprecipitate a platelet membrane complex consisting of proteins of 70, 90, 120, and 150 kDa (K Gong, GD MacFarlane, M Costalonga, P Liu, and MC Herzberg, 2001, unpublished data). These proteins and an additional protein antigen of 195 kDa were immunoprecipitated by Mab2.1. The 90 and 195 kDa proteins were identified as CD36 (GP IV) and CD31 (PECAM-1), respectively, by Western immunoblotting. The 120 kDa protein was immunoprecipitated by several anti-CD26 Mabs, which also reacted with platelets in flow cytometry. To isolate each protein, human platelet membranes were solubilized, and fractionated by Mab2 affinity chromatography followed by preparative SDS-PAGE. Isolated CD26 and CD31 inhibited platelet adhesion to *S. sanguis* by a maximum of 40 and 61%, respectively. Mixed together, these two proteins inhibited adhesion by about 81%. Adhesion of platelets was unaffected by incubation with isolated CD36 or α-actinin (protein control). Similarly, platelet adhesion was partially inhibited by preincubation with anti-CD26 or CD31 Mabs; anti-CD36 did not affect adhesion. Preincubation of platelet-rich plasma (PRP) with anti-CD31 Mab partially inhibited *S. sanguis*-induced aggregation; collagen-induced aggregation was virtually unaffected. Anti-CD36 completely inhibited *S. sanguis*-induced aggregation and delayed collagen induced aggregation 17-fold (0.2 nmole Mab/450 μl PRP). Anti-CD26 (11.7 nmole) and a tripeptide inhibitor of CD26-associated dipeptidyl peptidase IV activity also inhibited aggregation completely. CD26 and CD36, therefore, appear to mediate platelet aggregation in response to collagen, complementing GPVI and integrin-associated signal transduction pathways. Similarly, a platelet surface complex of CD26, 31 and 36 appears to interact with *S. sanguis* 133–79. CD26 and CD31 may mediate adhesion and, with CD36, the complex promotes *S. sanguis*-induced platelet aggregation.

Anti-infective platelet responses

Release of platelet microbicidal proteins (PMPs)

Platelet microbicidal proteins (PMPs) are small, cationic peptides that possess potent microbicidal (both bactericidal and fungicidal) activities and antiadherence properties against common bloodstream pathogens. PMPs include thrombin-induced PMP-1 (tPMP-1), PMP-2, and alpha granule-derived PMP[135–137]. Recently recognized homologues, thrombocidins-1 and 2, are active antimicrobial peptides formed as post-translational, basic C-terminal

Cambridge Books Online © Cambridge University Press, 2009

truncation products of certain CXC chemokines[138]. The PMPs are released from platelets by thrombin and other agonists and act in part by permeabilizing and altering staphylococcal transmembrane potential[137,139–141]. Bacterial resistance to PMPs (thrombocidins-1 and 2) appears to be an important virulence factor in infective endocarditis and DIC, in which viable bacteria become trapped within platelet aggregates or thrombi. Specific resistance to cationic PMP-1 is encoded in the staphylococcal multidrug resistance gene *qacA*[45].

The action of the PMPs is readily shown in vitro; PMPs appear active in vivo. PMPs are released and rapidly kill *Streptococcus sanguis* M99 after inducing secretion and aggregation of rabbit platelets in vitro[38]. Furthermore, rabbits made thrombocytopenic with an antiplatelet antibody develop more highly colonized experimental vegetations in the endocarditis model in response to *S. sanguis* M99, than rabbits given a non-immune serum. Platelet release of PMPs in vivo appears, therefore, to serve an anti-infective function against sensitive strains of bacteria. Since the PMPs can also reduce *Staphylococcus aureus* adherence to platelets in vitro, other antimicrobial mechanisms may act against some sensitive strains[142,143].

A survey of bacteria and their interactions with platelets

At least 30 species of bacteria have been shown to interact directly or indirectly to modify or alter platelet function. Major mechanisms and pathological examples have been highlighted. To consider the constellation of platelet-interactive strategies intrinsic to a bacterial species, *Staphylococcus aureus* and *Porphyromonas gingivalis*, Gram-positive and negative bacteria, respectively, will be considered in greater detail. Interactions between oral streptococci and platelets, also the focus of many reports, have been discussed in recent reviews[25,26,144] and will not be detailed here.

Staphylococcus aureus

Staphylococcus aureus interactions with platelets are well studied in vitro and in experimental animals. Clinical manifestations of platelet interactions with *S. aureus* include disseminated intravascular coagulation (DIC) and infective endocarditis. In vitro protein A-positive *S. aureus* cells stimulate release of serotonin and aggregation of human platelets[145]. Platelet aggregation in response to *S. aureus* is fibrinogen dependent[146]. Interactions are mediated by the IgG Fc (gClqR) receptor on platelets[101,147], which mediates Fc fragment-sensitive binding of *S. aureus*. It is unclear if gC1qR is involved in the signal transduction to elicit a platelet response. The platelet response to *S. aureus* is biphasic. While fibrinogen is required, the secondary wave of aggregation is independent of the RGD binding site of platelet glycoprotein IIb/IIIa[102].

S. aureus expresses a surface clumping factor, ClfA, which binds fibrinogen[148,149]. Platelet aggregating strains of *S. aureus* use ClfA in vitro to present fibrinogen to an undefined platelet receptor that facilitates aggregation. The surface components on *S. aureus* that are necessary and sufficient to induce aggregation of human platelets are contained in peptidoglycan and protein A[150]. When mixed, protein A and peptidoglycan from *S. aureus* will induce platelet aggregation, but soluble lipoteichoic acid (LTA) is inhibitory. Lipoteichoic acid from *S. aureus* appears to inhibit activation of protein kinase C and mobilization of calcium from intracellular stores[36,37].

When analysed quantitatively by flow cytometry, *S. aureus* binding to platelets was found to involve a multiplicity of ligand–receptor interactions[131]. Protein A also binds Von Willebrand factor[151]. Since platelet glycoprotein Ib binds Von Willebrand factor, protein A expressing *S. aureus* may also agglutinate platelets by multiple bridging via Von Willebrand factor. *S. aureus* also binds thrombospondin through a protein A-independent mechanism[152]. Secreted and rebound by activated platelets, thrombospondin, may also serve as a binding bridge for *S. aureus*. While several bridging proteins probably participate[153], fibrinogen interactions enable *S. aureus* to bind better to activated than resting platelets[124] and appears to be a major virulence determinant in experimental endocarditis[33]. Contact-activated platelets mediate attachment of *S. epidermidis* to hydrophobic polyethylene polymer surfaces in the presence of adsorbed, antiadhesive plasma proteins[154]. As the thrombus accumulates more activated platelets and enlarges, the efficiency of staphylococcal binding increases[155]. Bacteria are found increasingly on and within the thrombus even in the presence of the antibiotic, rifampin. Bacterial viability appears inessential to drive the aggregation of platelets into the enlarging thrombus.

Important virulence factors such as protein A, fibronectin-binding proteins and α-hemolysin are environmentally regulated by SarA[156]. *SarA* is regulated and may promote adhesion of *S. aureus* to fibrin-rich matrix proteins during experimental endocarditis[34]. Consequently, expression of key virulence factors may be coregulated and change in vivo during infection.

S. aureus surface proteins, therefore, interact with platelets to promote binding and thrombotic aggregation. Potentially antagonistic to these interactions, *S. aureus*

Cambridge Books Online © Cambridge University Press, 2009

also expresses several enzymes and toxins with the potential to digest or damage platelets in the hemostatic process. *S. aureus* V8 protease cleaves a variety of the cytoplasmic platelet proteins[157,158], platelet glycoprotein IIb/IIIa[159], Von Willebrand factor[160], and IgG[161]. Furthermore, staphylokinase, a clinically important enzyme that activates plasminogen, reverses collagen-induced aggregation by promoting fibrinolysis[162,163]. The fibrinolytic effect is antagonized by α2-antiplasmin, but staphylokinase does not induce platelet aggregation itself[164]. Staphylokinase has been engineered with an RGD sequence, a segment of fibrinopeptide A and sequence from hirudin[165]. This multi-functional antithrombotic, fibrinolytic agent showed promise in preliminary testing.

Platelets express antimicrobial proteins, which are effective against many strains of *S. aureus*[166]. The ability of platelets to bind *S. aureus* is directly associated with frequency and extent of aggregation, but independent of the resistance of *S. aureus* to platelet antimicrobial mechanisms, including the PMPs[131]. Clinical isolates of *S. aureus* and viridans streptococci from patients with infective endocarditis tend to be resistant to PMPs when compared to isolates from other bacteremic subjects[167]. The PMPs appear to kill or inhibit growth of *S. aureus* in vivo and resistance is associated with enhanced virulence in experimental endocarditis[136,168].

While platelet aggregation in the presence of aspirin or quinacrine is not substantially inhibited in response to *S. aureus* in vitro[169], antiplatelet agents may reduce the mass and extent of infection of platelet vegetations in experimental endocarditis in rabbits[170,171]. When rabbits were treated with aspirin and ticlopidine, a synergistic reduction in the mass of valvular vegetations and the severity of infection was noted in *S. aureus* experimental endocarditis[172]. Furthermore, aspirin pretreatment in experimental rabbits reduced septic embolic events[173]. *S. aureus* will also cause experimental endocarditis in dogs and the experimental vegetations have been shown to contain activated platelets that can be targeted with ^{99m}technetium-GPIIb/IIIa pharmacologic antagonists[174]. In experimental endocarditis, therefore, the development of the platelet vegetation is probably triggered by direct interactions with *S. aureus* and secondarily by tissue factor-mediated coagulation. Activation of platelets in response to generalized coagulation pathways would more likely be aspirin-sensitive than the aspirin-insensitive response to *S. aureus* shown in vitro.

Staphylococcal toxins may also contribute to coagulopathies. Toxin-rich fractions have been shown to induce platelet aggregation in vitro[175–177], while α-toxins reduce myocardial contractility and increase coronary vasoconstriction in isolated rat hearts[178]. In contrast, toxic shock syndrome toxin-1 (TSST-1) inhibits aggregation of human platelets in response to epinephrine, ADP or platelet aggregating factor (PAF), but shows no effect in response to thrombin, collagen, or calcium ionophore[179]. Strain-specific expression of staphylococcal toxins may then cause diverse coagulopathies.

Porphyromonas gingivalis

P. gingivalis is of interest because it expresses several important platelet-interactive strategies and may model other Gram-negative bacteria. Through interactions with *P. gingivalis*, platelets may behave as inflammatory and procoagulant cells, reflecting its pathogenicity in local infections such as periodontitis[180]. As speculated more recently, *P. gingivalis* may promote inflammation and microthrombosis in cardiovascular disease through several mechanisms including direct interactions with platelets[180a]. *P. gingivalis* and other procoagulant and platelet-interactive microorganisms, such as *Chlamydia pneumoniae*[181] and *Helicobacter pylorii*[89], are hypothesized to promote vascular inflammation and perhaps infection, reflecting contributions to total systemic pathogen burden[182,183].

P. gingivalis expresses a PAAP-cross-reactive antigen, which contributes to its ability to induce platelet secretion and aggregation in vitro[95,180]. Platelet aggregation in vitro is also triggered by *P. gingivalis* protease activity, which cleaves platelet surface proteins but does not mimic thrombin[184]. In vivo, coagulation may be initiated and prolonged by *P. gingivalis*. Arginine-specific cysteine proteinases expressed by *P. gingivalis* activate and consume protein C in plasma, which may contibute to a thrombotic tendency[74]. These proteinases also activate factor X in plasma, triggering coagulation[71]. Furthermore, LPS from *P. gingivalis* and other black-pigmented Bacteroides promote rapid platelet secretion and microthrombosis in the lungs and other tissues of the mouse, even more effectively than from *Salmonella* and other enteric pathogens[185]. Hence, *P. gingivalis*, which is strongly associated with local contained soft and hard tissue infections originating in the gingiva, shows substantial potential to behave as a circulatory pathogen resulting in platelet dysfunction and thrombosis.

Other bacterial species

Clinical decision-making about the potential for bacteria to cause platelet pathology will require a comprehensive overview, such that virtually all known platelet-interactive species are identified and characterized. Table 51.1 presents a comprehensive summary for reference. With scientific progress, this list will grow and need modification.

Cambridge Books Online © Cambridge University Press, 2009

Table 51.1. Other bacterial species and their interactions with platelets

Species	Whole cells	Surface macromolecules	Enzymes/toxins	Indirect mechanisms	Animals/humans
Streptococci, group A	Induced secretion, aggregation, ASA-sensitive, fibrinogen-dependent[186]	Peptidoglycan causes complement-dependent thrombolysis (rabbit, not human platelets)[187,188]; LTA binds specifically, inhibits collagen-induced aggregation, not secretion[35]			Infection of neonatal mice with cells or SPE + Freund's adjuvant causes activation/aggregation in vivo, models human lymphocutaneous lymph node syndrome[189]; human bacteremia leads to thrombocytopenia/mortality[190]
Group B	Type III induced secretion, aggregation[186], cation- cyclooxygenase- fibrinogen-dependent, trypsin-sensitive[191]; trypsin-sensitive component inhibits aggregation in response to collagen, ADP, thrombin>> epinephrine, ristocetin (arachidonic acid, no effect)[192]	Antibodies against type III-specific sialic acid blocked GBS-induced secretion, aggregation[193]			Cells induce indomethecin-insensitive thrombocytopenia (rabbits)[194]
Group C	Induced aggregation, cation- cyclooxygenase- ADP-dependent, protease-sensitive[195]	*S. milleri* surface proteins bind platelet-fibrin clots[196]	*S. constellatus* expresses thrombin-like activity[196]		
S. pneumoniae	Induced aggregation, cAMP-dependent, shortened clotting time of whole blood, PRP, PPP[197]	Polysaccharide produces procoagulant effects on PRP, blood, PPP[197]; type-specific antibodies form immune complex on cell wall inducing release of serotonin[198]; peptidoglycan induces complement-dependent thrombolysis (rabbit, not human)[187]		Teichoic acid acts as phosphorylcholine PAF-like proinflammatory mediator[199]	Trypsin-resistant surface component induces complement-independent thrombocytopenia (rabbits)[200]; meningitis induces thrombocytopenia associated with mortality[201]
Enterococci	Induced secretion, aggregation, ASA-insensitive[169]				
Lactobacillus	Induced aggregation, strain-specific, pronase-sensitive, RDGS-dependent[125,202,203]; platelet interactivity restored by passage through animal[202]				
Listeria	Induces secretion, aggregation (rat), release listericidin (aggregation unaffected)[204]; complexes with actin, profilin causing rearrangement[205,206]				

Cambridge Books Online © Cambridge University Press, 2009

Salmonella	*S. minnesota* PMP-resistant, *S. typhii* PMP-sensitive[207]	*S. minnesota* lipid A induces PKC-dependent secretion, aggregation[208–210]			*S. typhimurium* infection induces thrombocytopenia, DIC in 1–2 days (rhesus monkey)[211]; *S. minnesota* LPS induces DIC, biphasic thrombocytopenia, first wave complement-dependent (rabbits)[212]
Shigella flexneri			Shiga toxin binds specific glycosphingolipids (Luke blood group antigen)[213]		Shiga toxin associated with TTP, hemolytic uremic syndrome[214]
Helicobacter pylorii					Cells extracts induce activation, aggregation in gastric microvessels (humans, mice; rats)[89,215]; PAF-release contributes to aggregation (rats)[216]
Pasturella			Leukotoxin (RTX family repeats in toxin) activates aggregates at low doses, lyses (forms pores) at high doses (ruminents)[217–219], modulates adhesion[220]; O-sialoglycoprotein endoprotease cleaves GPIb, induces unresponsiveness to low doses of thrombin[130]		Infection induces platelet activation (cows)[217]
***Chlamydia* spp.**				Upregulates TF-procoagulant activity on endothelial cells promoting platelet adhesion[181]	
Escherichia coli	Hemolysin-positive strains activate PKC, increase $[Ca_I^+]$, 12-HETE, serotonin release, induce aggregation; hemolysin-negative strains activate PKC only[221]; *E. coli* cells induce aggregation in vitro, + TXA2 antagonist inhibits[222]	LPS activates NO/cGMP pathway, inhibits aggregation[223]; inhibits PKC pathway, PLC[116]; lipid A (LPS) activates PKC, serotonin release, induces aggregation[117]; LPS-induced aggregation may require other blood cells[224]; LPS-induced aggregation inhibited by triflavin[123]		LPS activates complement alternate pathway inducing lysis (rabbit)[225]	LPS induces DIC, modest thrombocytopenia (complement-independent)[212]; LPS induces serotonin release, thrombocytopenia, pulmonary congestion (BALB/c mice)[226]; LPS + TXA2 antagonist improves survival (shock), reduces thrombocytopenia, no pulmonary congestion (rats)[222]; LPS thrombocytopenia, pulmonary/liver congestion RDGS-sensitive (baboon)[123]; LPS + NO inhibitor increases pulmonary/renal congestion, intravascular congestion (pig)[227]

Cambridge Books Online © Cambridge University Press, 2009

Table 51.1. (*cont.*)

Species	Whole cells	Surface macromolecules	Enzymes/toxins	Indirect mechanisms	Animals/humans
Borrelia burgdorferi	Aggregation-negative[228]; bind platelets via αIIbβ3[128,229]				αIIbβ3-dependent binding associated with virulence in humans[230]
Neisseria meningitidis					Sepsis causes microvascular thrombosis; LPS induces DIC[231,232]; DIC associated with CD14, TF^{+} platelet/granulocyte microparticles (generate thrombin in vitro)[88]
Clostridium perfringens			Δ-toxin attacks GM2-like gangliosides causing lysis[233]; PLC induces aggregation[104,113], ADP- and TXA2– independent, cAMP-dependent serotonin release[234], Ca^{2+} and PI hydrolysis-dependent[235]; platelet-leukocyte aggregation mediated by GPIIb/IIIa upregulation[236]		Aggregation in mesentary (rat)[237]
C. welchii			PLC induces aggregation, phosphatidic acid production[238], TXA2-independent aggregation, phosphatidic acid mobilizes Ca^{2+}[239]		
C. botulinum			ADP-ribosyl-transferase C3 ADP-ribosylates 21–24 kDa GTP-binding proteins[240] identified as membrane and cytosol RhoA[241], which regulates actin assembly, GPIIb/IIIa avidity during aggregation[242], alters integrin ligation to actin[243], inhibits myosin light chain phosphorylation and shape change (blocks rho/rho-kinase-dependent regulation of MLC phosphorylation[244, 245]		

Cambridge Books Online © Cambridge University Press, 2009

C. difficile					Toxin A induces platelet-leukocyte aggregates in microvasculature, up-regulates P-selectin (rat)[246]
Pseudomonas aeruginosa	Induces aggregation[27]; complement-independent secretion[247]		Lipase + phospholipase C catalyses 12-HETE release[248,249]; PLC induces aggregation[105]		Disrupted cells induce aggregation, thrombocytopenia, pulmonary microthrombi in vivo (dogs)[250]
Bacillus subtillus	Rapidly killed by thrombocidin (PMP)[138,251]				PLC^{+} strain induces thrombocytopenia, intravascular coagulation, pulmonary congestion[252]
B. cereus			Exoenzyme ADP-ribosylates 20–25 kDa GTP binding membrane protein (similar to exoenzymes from *C. botulinum*, *C. limnosum*)[253]		
B. thuringiensis			PI-specific PLC induces release of glycoprotein fragment of GPIb complex[254]		
Flavobacterium ssp		13-methyl-myristate (soluble) induces lysis, perturbation[255]		Sulfobacins A and B inhibit binding of VWF to receptor, ristocetin-induced aggregation[256]	
Fusobacterium necrophorum	Virulent strains induce serotonin release, aggregation[257]; aggregation associated with hemagglutination activity[258], biovar A[259]	LPS biovar A induces aggregation (bovine)[259]			
Serratia marcesens	Inhibits aggregation of fresh, washed platelets[260,261]		Extracellular Zn^{2+}-metallo-protease cleaves GPIb, not GPV, attenuates responses to thrombin, VWF[262–264]		LPS induces DIC with biphasic thrombocytopenia, phase 1 complement-dependent (rabbits)[212]
Prevotella intermedia					LPS induces accumulation in lungs, rapid release of serotonin (mice)[185]
Klebsiella pneumoniae					LPS (low dose) induces complement-independent accumulation in lungs, complement-dependent anaphylactoid shock (mice)[273]; cells induce DIC, shock death; course improved by hirudin (rats)[265,266]

Cambridge Books Online © Cambridge University Press, 2009

Table 51.1. (*cont.*)

Species	Whole cells	Surface macromolecules	Enzymes/toxins	Indirect mechanisms	Animals/humans
Bordetella pertussis			Adenylate cyclase toxin (RTX family) cytolytic[219]; increases cAMP inhibiting ADP-induced aggregation (rabbit)[267]		
Yersinia pestis		YopM (homology to thrombin-binding site of GPIb) inhibits thrombin-induced aggregation[268]			
Y. pseudo-tuberculosis	Induces aggregation[269]	Inv (103 kDa) interacts with GPIcIIa[269]			
Leptospira		LPS (strain variability) induces secretion, LPS (few strains) induce aggregation[270]		LPS induces PAF release from PMNs mediating aggregation[271]	Thrombocytopenia (DIC-independent) associated with activated endothelium promoting adhesion, aggregation[272]

Cambridge Books Online © Cambridge University Press, 2009

Acknowledgements

Work in the authors' laboratory investigating platelet–bacterial interactions was conducted with the support of grants from the National Institutes of Health (DE05501, DE00224, DE00270, and DE08590). Thanks are due to many wonderful students, fellows, and technicians who asked provocative questions, to Professor Emeritus Maurice W. Meyer for establishing in vivo real-time models to study interactions, and C. C. Clawson, Professor of Pediatrics, who introduced MCH to the platelet in the context of infection.

References

1 Clawson CC. Platelets in Bacterial Infections. In: Joseph M, ed. *Immunopharmacology of platelets* London: Academic Press Limited; 1995: 83–124.
2 Fishbein DB, Dawson JE, Robinson LE. Human ehrlichiosis in the United States, 1985 to 1990. *Ann Intern Med* 1994; 120: 736–743.
3 Jerrard D. Ehrlichiosis. *J Emerg Med* 1999; 17: 27–30.
4 Ogden NH, Woldehiwet Z, Hart CA. Granulocytic ehrlichiosis: an emerging or rediscovered tick-borne disease? *J Med Microbiol* 1998; 47: 475–482.
5 Schutze GE, Jacobs RF. Human monocytic ehrlichiosis in children. *Pediatrics* 1997; 100: E10.
6 Dumler JS, Dawson JE, Walker DH. Human ehrlichiosis: hematopathology and immunohistologic detection of *Ehrlichia chaffeensis. Hum Pathol* 1993; 24: 391–396.
7 Walls JJ, Aguero-Rosenfeld M, Bakken JS et al. Inter- and intralaboratory comparison of *Ehrlichia equi* and human granulocytic ehrlichiosis (HGE) agent strains for serodiagnosis of HGE by the immunofluorescent-antibody test. *J Clin Microbiol* 1999; 37: 2968–2973.
8 Bakken JS. The discovery of human granulocytotropic ehrlichiosis. *J Lab Clin Med* 1998;132:175–180.
9 Comer JA, Tzianabos T, Flynn C, Vlahov D, Childs JE. Serologic evidence of rickettsialpox (*Rickettsia akari*) infection among intravenous drug users in inner-city Baltimore, Maryland. *Am J Trop Med Hyg* 1999; 60: 894–898.
10 Mathew JS, Ewing SA, Murphy GL, Kocan KM, Corstvet RE, Fox JC. Characterization of a new isolate of *Ehrlichia platys* (Order Rickettsiales) using electron microscopy and polymerase chain reaction. *Vet Parasitol* 1997; 68: 1–10.
11 Walls JJ, Caturegli P, Bakken JS, Asanovich KM, Dumler JS. Improved sensitivity of PCR for diagnosis of human granulocytic ehrlichiosis using epank1 genes of *Ehrlichia phagocytophila*-group ehrlichiae. *J Clin Microbiol* 2000; 38: 354–356.
12 Hoilien CA, Ristic M, Huxsoll DL, Rapmund G. *Rickettsia sennetsu* in human blood monocyte cultures: similarities to the growth cycle of *Ehrlichia canis. Infect Immun* 1982; 35: 314–319.
13 Elghetany MT, Walker DH. Hemostatic changes in Rocky Mountain spotted fever and Mediterranean spotted fever. *Am J Clin Pathol* 1999; 112: 159–168.
14 Buller RS, Arens M, Hmiel SP, Paddock CD, Sumner JW, Rikhisa Y et al. *Ehrlichia ewingii*, a newly recognized agent of human ehrlichiosis. *N Engl J Med* 1999; 341: 148–155.
15 Rapmund G. Rickettsial diseases of the Far East: new perspectives. *J Infect Dis* 1984; 149: 330–338.
16 Rikihisa Y. Clinical and biological aspects of infection caused by *Ehrlichia chaffeensis. Microbes Infect* 1999; 1: 367–376.
17 Wong SJ, Thomas JA. Cytoplasmic, nuclear, and platelet autoantibodies in human granulocytic ehrlichiosis patients. *J Clin Microbiol* 1998; 36: 1959–1963.
18 Grindem CB, Breitschwerdt EB, Perkins PC, Cullins LD, Thomas TJ, Hegarty BC. Platelet-associated immunoglobulin (antiplatelet antibody) in canine Rocky Mountain spotted fever and ehrlichiosis. *J Am Anim Hosp Assoc* 1999; 35: 56–61.
19 Kakoma I, Carson CA, Ristic M, Stephenson EM, Hildebrandt PK, Huxsoll DL. Platelet migration inhibition as an indicator of immunologically mediated target cell injury in canine ehrlichiosis. *Infect Immun* 1978; 20: 242–247.
20 Gaunt SD, Baker DC, Babin SS. Platelet aggregation studies in dogs with acute *Ehrlichia platys* infection. *Am J Vet Res* 1990; 51: 290–293.
21 Harrus S, Waner T, Eldor A, Zwang E, Bark H. Platelet dysfunction associated with experimental acute canine ehrlichiosis. *Vet Rec* 1996; 139: 290–293.
22 Pierce KR, Marrs GE, Hightower D. Acute canine ehrlichiosis: platelet survival and factor 3 assay. *Am J Vet Res* 1977; 38: 1821–1825.
23 Durack DT. Prevention of infective endocarditis. *N Engl J Med* 1995; 332: 38–44.
24 Dyson C, Barnes RA, Harrison GA. Infective endocarditis: an epidemiological review of 128 episodes. *J Infect* 1999; 38: 87–93.
25 Herzberg MC. Persistence of infective endocarditis. In: Nataro JP, Blaser MJ, Cunningham-Rundkes S, eds. *Persistent bacterial infections.* Washington, DC: ASM Press; 2000: 357–374.
26 Herzberg MC. Platelet-streptococcal interactions in endocarditis. *Crit Rev Oral Biol Med* 1996; 7: 222–236.
27 Kessler CM, Nussbaum E, Tuazon CU. In vitro correlation of platelet aggregation with occurrence of disseminated intravascular coagulation and subacute bacterial endocarditis. *J Lab Clin Med* 1987; 109: 647–652.
28 Gong K, Ouyang T, Herzberg MC. A streptococcal adhesion system for salivary pellicle and platelets. *Infect Immun* 1998; 66: 5388–5392.
29 Herzberg MC, Brintzenhofe KL, Clawson CC. Cell-free released components of *Streptococcus sanguis* inhibit human platelet aggregation. *Infect Immun* 1983; 42: 394–401.
30 Herzberg MC, Brintzenhofe KL, Clawson CC. Aggregation of human platelets and adhesion of *Streptococcus sanguis. Infect Immun* 1983; 39: 1457–1469.
31 Gong K, Wen DY, Ouyang T, Rao AT, Herzberg MC. Platelet

Cambridge Books Online © Cambridge University Press, 2009

receptors for the *Streptococcus sanguis* adhesin and aggregation-associated antigens are distinguished by anti-idiotypical monoclonal antibodies. *Infect Immun* 1995; 63: 3628–3633.

32 Soberay AH, Herzberg MC, Rudney JD, Nieuwenhuis HK, Sixma JJ, Seligsohn U. Responses of platelets to strains of *Streptococcus sanguis*: findings in healthy subjects, Bernard–Soulier, Glanzmann's, and collagen-unresponsive patients. *Thromb Haemost* 1987; 57: 222–225.

33 Sullam PM, Bayer AS, Foss WM, Cheung AL. Diminished platelet binding in vitro by *Staphylococcus aureus* is associated with reduced virulence in a rabbit model of infective endocarditis. *Infect Immun* 1996; 64: 4915–4921.

34 Cheung AL, Yeaman MR, Sullam PM, Witt MD, Bayer AS. Role of the sar locus of *Staphylococcus aureus* in induction of endocarditis in rabbits. *Infect Immun* 1994; 62: 1719–1725.

35 Beachey EH, Chiang TM, Ofek I, Kang AH. Interaction of lipoteichoic acid of group A streptococci with human platelets. *Infect Immun* 1977; 16: 649–654.

36 Sheu JR, Hsiao G, Lee C et al. Antiplatelet activity of *Staphylococcus aureus* lipoteichoic acid is mediated through a cyclic AMP pathway. *Thromb Res* 2000; 99: 249–258.

37 Sheu JR, Lee CR, Lin CH, Hsiao G, Ko WC, Chen YC et al. Mechanisms involved in the antiplatelet activity of *Staphylococcus aureus* lipoteichoic acid in human platelets. *Thromb Haemost* 2000; 83: 777–784.

38 Sullam PM, Costerton JW, Yamasaki R, Dazin PF, Mills J. Inhibition of platelet binding and aggregation by streptococcal exopolysaccharide. *J Infect Dis* 1993; 167: 1123–1130.

39 Yeaman MR, Bayer AS. *Staphylococcus aureus*, platelets, and the heart. *Curr Infect Dis Rep* 2000; 2: 281–298.

40 Bayer AS, Bolger AF, Taubert KA et al. Diagnosis and management of infective endocarditis and its complications. *Circulation* 1998; 98: 2936–2948.

41 Olaison L, Hogevik H, Alestig K. Fever, C-reactive protein, and other acute-phase reactants during treatment of infective endocarditis. *Arch Intern Med* 1997; 157: 885–892.

42 Wells AU, Fowler CC, Ellis-Pegler RB, Luke R, Hannan S, Sharpe DN. Endocarditis in the 80s in a general hospital in Auckland, New Zealand. *Q J Med* 1990; 76: 753–762.

43 Taha TH, Durrant S, Crick J, Bowcock S, Bradshaw A, Oakley CM. Hemostatic studies in patients with infective endocarditis: a report on nine consecutive cases with evidence of coagulopathy. *Heart Vessels* 1991; 6: 102–106.

44 Fukuda Y, Kuroiwa Y, Tabuchi H et al. A thrombotic tendency in patients with infective endocarditis. *Jpn Circ J* 1982; 46: 460–467.

45 Kupferwasser LI, Skurray RA, Brown MH, Firth N, Yeaman MR, Bayer AS. Plasmid-mediated resistance to thrombin-induced platelet microbicidal protein in staphylococci: role of the *qacA* locus. *Antimicrob Agents Chemother* 1999; 43: 2395–2399.

46 Mayo JA, Zhu H, Harty DW, Knox KW. Modulation of glycosidase and protease activities by chemostat growth conditions in an endocarditis strain of *Streptococcus sanguis*. *Oral Microbiol Immunol* 1995; 10: 342–348.

47 Drake TA, Pang M. Effects of interleukin-1, lipopolysaccharide, and streptococci on procoagulant activity of cultured human cardiac valve endothelial and stromal cells. *Infect Immun* 1989; 57: 507–512.

48 Bancsi MJ, Thompson J, Bertina RM. Stimulation of monocyte tissue factor expression in an in vitro model of bacterial endocarditis. *Infect Immun* 1994; 62: 5669–5572.

49 Bancsi MJ, Veltrop MH, Bertina RM, Thompson J. Influence of monocytes and antibiotic treatment on tissue factor activity of endocardial vegetations in rabbits infected with *Streptococcus sanguis*. *Infect Immun* 1996; 64: 448–451.

50 Douglas CW, Brown PR, Preston FE. Platelet aggregation by oral streptococci. *FEMS Microbiol Lett* 1990; 60: 63–67.

51 Herzberg MC, Gong K, MacFarlane GD et al. Phenotypic characterization of *Streptococcus sanguis* virulence factors associated with bacterial endocarditis. *Infect Immun* 1990; 58: 515–522.

52 Sullam PM, Valone FH, Mills J. Mechanisms of platelet aggregation by viridans group streptococci. *Infect Immun* 1987; 55: 1743–1750.

53 Johnson CM, Bowie EJ. Pigs with von Willebrand disease may be resistant to experimental infective endocarditis. *J Lab Clin Med* 1992; 120: 553–558.

54 Herzberg MC, MacFarlane GD, Gong K et al. The platelet interactivity phenotype of *Streptococcus sanguis* influences the course of experimental endocarditis. *Infect Immun* 1992; 60: 4809–4818.

55 Straus DC, Mattingly SJ, Milligan TW. Production of extracellular material by streptococci associated with subacute bacterial endocarditis. *Infect Immun* 1977; 17: 148–156.

56 Gutschik E, Moller S, Christensen N. Experimental endocarditis in rabbits. 3. Significance of the proteolytic capacity of the infecting strains of *Streptococcus faecalis*. *Acta Pathol Microbiol Scand [B]* 1979; 87: 353–362.

57 Levi M, Ten Cate H. Disseminated intravascular coagulation. *N Engl J Med* 1999; 341: 586–592.

58 Meyer MW, Gong K, Herzberg MC. *Streptococcus sanguis*-induced platelet clotting in rabbits and hemodynamic and cardiopulmonary consequences. *Infect Immun* 1998; 66: 5906–5914.

59 Briede JJ, Tans G, Willems GM, Hemker HC, Lindhout T. Regulation of platelet factor Va-dependent thrombin generation by activated protein C at the surface of collagen-adherent platelets. *J Biol Chem* 2000; 11: 11.

60 Dormann D, Clemetson KJ, Kehrel BE. The GPIb thrombin-binding site is essential for thrombin-induced platelet procoagulant activity. *Blood* 2000; 96: 2469–2478.

61 Hardaway RM. A review of septic shock. *Am Surg* 2000; 66: 22–29.

62 Levi M, de Jonge E, van der Poll T, ten Cate H. Novel approaches to the management of disseminated intravascular coagulation. *Crit Care Med* 2000; 28: S20–S24.

63 Mammen EF. The haematological manifestations of sepsis. *J Antimicrob Chemother* 1998; 41: 17–24.

64 Levi M, de Jonge E. Current management of disseminated intravascular coagulation. *Hosp Pract (Off Ed)* 2000; 35: 59–66; quiz 92.

65 Mertens R, Peschgens T, Granzen B, Heimann G. Diagnosis

Cambridge Books Online © Cambridge University Press, 2009

and stage-related treatment of disseminated intravascular coagulation in meningococcal infections. *Klin Pediatr* 1999; 211: 65–69.
66 Wada H, Gabazza E, Nakasaki T et al. Diagnosis of disseminated intravascular coagulation by hemostatic molecular markers. *Semin Thromb Hemost* 2000; 26: 17–21.
67 Wada H, Sakuragawa N, Mori Y et al. Hemostatic molecular markers before the onset of disseminated intravascular coagulation. *Am J Hematol* 1999; 60: 273–278.
68 Yu M, Nardella A, Pechet L. Screening tests of disseminated intravascular coagulation: guidelines for rapid and specific laboratory diagnosis. *Crit Care Med* 2000; 28: 1777–1780.
69 Folman CC, Linthorst GE, van Mourik J et al. Platelets release thrombopoietin (TPO) upon activation: another regulatory loop in thrombocytopoiesis? *Thromb Haemost* 2000; 83: 923–930.
70 Collen D. Staphylokinase: a potent, uniquely fibrin-selective thrombolytic agent. *Nat Med* 1998; 4: 279–284.
71 Imamura T, Potempa J, Tanase S, Travis J. Activation of blood coagulation factor X by arginine-specific cysteine proteinases (gingipain-Rs) from *Porphyromonas gingivalis. J Biol Chem* 1997; 272: 16062–16067.
72 Donabedian H, Boyle MD. Clot formation by group A streptococci. *Infect Immun* 1998; 66: 2362–2364.
73 Matsuka YV, Pillai S, Gubba S, Musser JM, Olmsted SB. Fibrinogen cleavage by the *Streptococcus pyogenes* extracellular cysteine protease and generation of antibodies that inhibit enzyme proteolytic activity. *Infect Immun* 1999; 67: 4326–4333.
74 Hosotaki K, Imamura T, Potempa J, Kitamura N, Travis J. Activation of protein C by arginine-specific cysteine proteinases (gingipains-R) from *Porphyromonas gingivalis. Biol Chem* 1999; 380: 75–80.
75 Colman RW. The contact system: a proinflammatory pathway with antithrombotic activity. *Nat Med* 1998; 4: 277–278.
76 Herwald H, Morgelin M, Olsen A et al. Activation of the contact-phase system on bacterial surfaces—a clue to serious complications in infectious diseases. *Nat Med* 1998; 4: 298–302.
77 Mavrommatis AC, Theodoridis T, Orfanidou A, Roussos C, Christopoulou-Kokkinou V, Zakynthinos S. Coagulation system and platelets are fully activated in uncomplicated sepsis. *Crit Care Med* 2000; 28: 451–457.
78 Libman H, Arbeit RD. Complications associated with *Staphylococcus aureus* bacteremia. *Arch Intern Med* 1984; 144: 541–545.
79 O'Connor DT, Weisman MH, Fierer J. Activation of the alternate complement pathway in *Staph. aureus* infective endocarditis and its relationship to thrombocytopenia, coagulation abnormalities, and acute glomerulonephritis. *Clin Exp Immunol* 1978; 34: 179–187.
80 Arning M, Gehrt A, Aul C, Runde V, Hadding U, Schneider W. Septicemia due to *Streptococcus mitis* in neutropenic patients with acute leukemia. *Blut* 1990; 61: 364–368.
81 Bochud PY, Calandra T, Francioli P. Bacteremia due to viridans streptococci in neutropenic patients: a review. *Am J Med* 1994; 97: 256–264.
82 Elting LS, Bodey GP, Keefe BH. Septicemia and shock syndrome due to viridans streptococci: a case-control study of predisposing factors. *Clin Infect Dis* 1992; 14: 1201–1207.
83 Rintala E, Kauppila M, Seppala OP et al. Protein C substitution in sepsis-associated purpura fulminans. *Crit Care Med* 2000; 28: 2373–2378.
84 Gikas A, Samonis G, Christidou A et al. Gram-negative bacteremia in non-neutropenic patients: a 3-year review. *Infection* 1998; 26: 155–159.
85 Herf C, Nichols J, Fruh S, Holloway B, Anderson CU. Meningococcal disease: recognition, treatment, and prevention. *Nurse Pract* 1998; 23: 30, 33–6, 39–40 passim.
86 ten Cate H. Pathophysiology of disseminated intravascular coagulation in sepsis. *Crit Care Med* 2000; 28: S9–S11.
87 Lundahl TH, Lindahl TL, Fagerberg IH, Egberg N, Bunescu A, Larsson A. Activated platelets and impaired platelet function in intensive care patients analyzed by flow cytometry. *Blood Coagul Fibrinol* 1996; 7: 218–220.
88 Nieuwland R, Berckmans RJ, McGregor S et al. Cellular origin and procoagulant properties of microparticles in meningococcal sepsis. *Blood* 2000; 95: 930–935.
89 Elizalde JI, Gomez J, Panes J et al. Platelet activation in mice and human *Helicobacter pylori* infection. *J Clin Invest* 1997; 100: 996–1005.
90 Bick RL. Syndromes of disseminated intravascular coagulation in obstetrics, pregnancy, and gynecology. Objective criteria for diagnosis and management. *Hematol Oncol Clin North Am* 2000; 14: 999–1044.
91 Bick RL. Disseminated intravascular coagulation: pathophysiological mechanisms and manifestations. *Semin Thromb Haemost* 1998; 24: 3–18.
92 Esmon CT, Fukudome K, Mather T et al. Inflammation, sepsis, and coagulation. *Haematologica* 1999; 84: 254–259.
93 Meyer MW, Witt AR, Krishnan LK et al. Therapeutic advantage of recombinant human plasminogen activator in endocarditis: evidence from experiments in rabbits. *Thromb Haemost* 1995; 73: 680–682.
94 Erickson PR, Herzberg MC. Evidence for the covalent linkage of carbohydrate polymers to a glycoprotein from *Streptococcus sanguis. J Biol Chem* 1993; 268: 23780–23783.
95 Erickson PR, Herzberg MC. The *Streptococcus sanguis* platelet aggregation-associated protein. Identification and characterization of the minimal platelet-interactive domain. *J Biol Chem* 1993; 268: 1646–1649.
96 Erickson PR, Herzberg MC. A collagen-like immunodeterminant on the surface of *Streptococcus sanguis* induces platelet aggregation. *J Immunol 1*987; 138: 3360–3366.
97 Lukomski S, Nakashima K, Abdi I et al. Identification and characterization of the *scl* gene encoding a group A Streptococcus extracellular protein virulence factor with similarity to human collagen. *Infect Immun* 2000; 68: 6542–6553.
98 Erickson PR, Herzberg MC. Altered expression of the platelet aggregation-associated protein from *Streptococcus sanguis* after growth in the presence of collagen. *Infect Immun* 1995; 63: 1084–1088.

Cambridge Books Online © Cambridge University Press, 2009

99 Kilic AO, Herzberg MC, Meyer MW, Zhao X, Tao L. Streptococcal reporter gene-fusion vector for identification of in vivo expressed genes. *Plasmid* 1999; 42: 67–72.
100 Graille M, Stura EA, Corper AL et al. Crystal structure of a *Staphylococcus aureus* protein A domain complexed with the Fab fragment of a human IgM antibody: structural basis for recognition of B-cell receptors and superantigen activity. *Proc Natl Acad Sci USA* 2000; 97: 5399–5404.
101 Nguyen T, Ghebrehiwet B, Peerschke EI. *Staphylococcus aureus* protein A recognizes platelet gC1qR/p33: a novel mechanism for staphylococcal interactions with platelets. *Infect Immun* 2000; 68: 2061–2068.
102 Bayer AS, Sullam PM, Ramos M, Li C, Cheung AL, Yeaman MR. *Staphylococcus aureus* induces platelet aggregation via a fibrinogen-dependent mechanism which is independent of principal platelet glycoprotein IIb/IIIa fibrinogen-binding domains. *Infect Immun* 1995; 63: 3634–3641.
103 Marques MB, Weller PF, Parsonnet J, Ransil BJ, Nicholson-Weller A. Phosphatidylinositol-specific phospholipase C, a possible virulence factor of *Staphylococcus aureus*. *J Clin Microbiol* 1989; 27: 2451–2454.
104 Sugahara T, Takahashi T, Yamaya S, Ohsaka A. In vitro aggregation of platelets induced by alpha-toxin (phospholipase C) of *Clostridium perfringens*. *Jpn J Med Sci Biol* 1976; 29: 255–263.
105 Coutinho IR, Berk RS, Mammen E. Platelet aggregation by a phospholipase C from *Pseudomonas aeruginosa*. *Thromb Res* 1988; 51: 495–505.
106 Barzaghi G, Cerletti C, de Gaetano G. Phospholipase C from *Clostridium perfringens* induces human platelet aggregation in plasma. *Thromb Haemost* 1988; 59: 236–239.
107 Leslie D, Fairweather N, Pickard D, Dougan G, Kehoe M. Phospholipase C and haemolytic activities of *Clostridium perfringens* alpha-toxin cloned in *Escherichia coli*: sequence and homology with a *Bacillus cereus* phospholipase C. *Mol Microbiol* 1989; 3: 383–392.
108 Olofsson A, Kaveus U, Thelestam M, Hebert H. The projection structure of alpha-toxin from *Staphylococcus aureus* in human platelet membranes as analyzed by electron microscopy and image processing. *J Ultrastruct Mol Struct Res* 1988; 100: 194–200.
109 Titball RW, Hunter SE, Martin KL et al. Molecular cloning and nucleotide sequence of the alpha-toxin (phospholipase C) of *Clostridium perfringens*. *Infect Immun* 1989; 57: 367–376.
110 Sharom FJ, McNeil GL, Glover JR, Seier S. Modulation of the cleavage of glycosylphosphatidylinositol-anchored proteins by specific bacterial phospholipases. *Biochem Cell Biol* 1996; 74: 701–713.
111 Anwer K, Wallace D, Romstedt K, Huzoor A. Human platelet activation by bacterial phospholipase C: mechanism of inhibition by flurazepam. *Thromb Res* 1988; 49: 225–239.
112 Guillouard I, Alzari PM, Saliou B, Cole ST. The carboxy-terminal C2-like domain of the alpha-toxin from *Clostridium perfringens* mediates calcium-dependent membrane recognition. *Mol Microbiol* 1997; 26: 867–876.
113 Otnaess AB, Holm T. The effect of phospholipase C on human blood platelets. *J Clin Invest* 1976; 57: 1419–1425.
114 Ikigai H, Nakae T. Assembly of the alpha-toxin-hexamer of *Staphylococcus aureus* in the liposome membrane. *J Biol Chem* 1987; 262: 2156–2160.
115 Fink D, Contreras ML, Lelkes PI, Lazarovici P. *Staphylococcus aureus* alpha-toxin activates phospholipases and induces a Ca^{2+} influx in PC12 cells. *Cell Signal* 1989; 1: 387–393.
116 Sheu JR, Hung WC, Kan YC, Lee YM, Yen MH. Mechanisms involved in the antiplatelet activity of *Escherichia coli* lipopolysaccharide in human platelets. *Br J Haematol* 1998; 103: 29–38.
117 Grabarek J, Timmons S, Hawiger J. Modulation of human platelet protein kinase C by endotoxic lipid A. *J Clin Invest* 1988; 82: 964–971.
118 Ghebrehiwet B, Peerschke EI. Structure and function of gC1q-R: a multiligand binding cellular protein. *Immunobiology* 1998; 199: 225–238.
119 Ghebrehiwet B, Peerschke EI, Hong Y, Munoz P, Gorevic PD. Short amino acid sequences derived from C1q receptor (C1q-R) show homology with the alpha chains of fibronectin and vitronectin receptors and collagen type IV. *J Leukoc Biol* 1992; 51: 546–556.
120 Peerschke EI, Reid KB, Ghebrehiwet B. Platelet activation by C1q results in the induction of alpha IIb/beta 3 integrins (GPIIb–IIIa) and the expression of P-selectin and procoagulant activity. *J Exp Med* 1993; 178: 579–587.
121 Peerschke EI, Reid KB, Ghebrehiwet B. Identification of a novel 33-kDa C1q-binding site on human blood platelets. *J Immunol* 1994; 152: 5896–5901.
122 Lu PD, Galanakis DK, Ghebrehiwet B, Peerschke EI. The receptor for the globular 'heads' of C1q, gC1q-R, binds to fibrinogen/fibrin and impairs its polymerization. *Clin Immunol* 1999; 90: 360–367.
123 Taylor FB, Coller BS, Chang AC et al. 7E3 F(ab′)2, a monoclonal antibody to the platelet GPIIb/IIIa receptor, protects against microangiopathic hemolytic anemia and microvascular thrombotic renal failure in baboons treated with C4b binding protein and a sublethal infusion of *Escherichia coli*. *Blood* 1997; 89: 4078–4084.
124 Herrmann M, Lai QJ, Albrecht RM, Mosher DF, Proctor RA. Adhesion of *Staphylococcus aureus* to surface-bound platelets: role of fibrinogen/fibrin and platelet integrins. *J Infect Dis* 1993; 167: 312–322.
125 Harty DW, Patrikakis M, Hume EB, Oakey HJ, Knox KW. The aggregation of human platelets by *Lactobacillus* species. *J Gen Microbiol* 1993; 139: 2945–2951.
126 Ford I, Douglas CW, Cox D, Rees DG, Heath J, Preston FE. The role of immunoglobulin G and fibrinogen in platelet aggregation by *Streptococcus sanguis*. *Br J Haematol* 1997; 97: 737–746.
127 Stockbauer KE, Magoun L, Liu M et al. A natural variant of the cysteine protease virulence factor of group A *Streptococcus* with an arginine-glycine-aspartic acid (RGD) motif preferentially binds human integrins alphavbeta3 and alphaIIbbeta3. *Proc Natl Acad Sci USA* 1999; 96: 242–247.

Cambridge Books Online © Cambridge University Press, 2009

128 Coburn J, Leong JM, Erban JK. Integrin alpha IIb beta 3 mediates binding of the Lyme disease agent *Borrelia burgdorferi* to human platelets. *Proc Natl Acad Sci USA* 1993; 90: 7059–7063.

129 Ford I, Douglas CW, Preston FE, Lawless A, Hampton KK. Mechanisms of platelet aggregation by *Streptococcus sanguis*, a causative organism in infective endocarditis. *Br J Haematol* 1993; 84: 95–100.

130 Kinlough-Rathbone RL, Perry DW, Rand ML, Packham MA. Responses to aggregating agents after cleavage of GPIb of human platelets by the o-sialoglycoprotein endoprotease from *Pasteurella haemolytica* – potential surrogates for Bernard–Soulier platelets? *Thromb Res* 2000; 99: 165–172.

131 Yeaman MR, Norman DC, Bayer AS. *Staphylococcus aureus* susceptibility to thrombin-induced platelet microbicidal protein is independent of platelet adherence and aggregation in vitro. *Infect Immun* 1992; 60: 2368–2374.

132 Jandrot-Perrus M, Busfield S, Lagrue AH et al. Cloning, characterization, and functional studies of human and mouse glycoprotein VI: a platelet-specific collagen receptor from the immunoglobulin superfamily. *Blood* 2000; 96: 1798–1807.

133 Gross BS, Melford SK, Watson SP. Evidence that phospholipase C-gamma2 interacts with SLP-76, Syk, Lyn, LAT and the Fc receptor gamma-chain after stimulation of the collagen receptor glycoprotein VI in human platelets. *Eur J Biochem* 1999; 263: 612–623.

134 Nieswandt B, Bergmeier W, Schulte V, Rackebrandt K, Gessner JE, Zirngibl H. Expression and function of the mouse collagen receptor glycoprotein VI is strictly dependent on its association with the FcRgamma chain. *J Biol Chem* 2000; 275: 23998–24002.

135 Bayer AS, Prasad R, Chandra J et al. In vitro resistance of *Staphylococcus aureus* to thrombin-induced platelet microbicidal protein is associated with alterations in cytoplasmic membrane fluidity. *Infect Immun* 2000; 68: 3548–3553.

136 Dhawan VK, Yeaman MR, Cheung AL, Kim E, Sullam PM, Bayer AS. Phenotypic resistance to thrombin-induced platelet microbicidal protein in vitro is correlated with enhanced virulence in experimental endocarditis due to *Staphylococcus aureus*. *Infect Immun* 1997; 65: 3293–3299.

137 Yeaman MR, Bayer AS, Koo SP, Foss W, Sullam PM. Platelet microbicidal proteins and neutrophil defensin disrupt the *Staphylococcus aureus* cytoplasmic membrane by distinct mechanisms of action. *J Clin Invest* 1998; 101: 178–187.

138 Krijgsveld J, Zaat SA, Meeldijk J, van Veelen PA, Fang G, Poolman B et al. Thrombocidins, microbicidal proteins from human blood platelets, are C-terminal deletion products of CXC chemokines. *J Biol Chem* 2000; 275: 20374–20381.

139 Koo SP, Bayer AS, Kagan BL, Yeaman MR. Membrane permeabilization by thrombin-induced platelet microbicidal protein 1 is modulated by transmembrane voltage polarity and magnitude. *Infect Immun* 1999; 67: 2475–2481.

140 Koo SP, Bayer AS, Sahl HG, Proctor RA, Yeaman MR. Staphylocidal action of thrombin-induced platelet microbicidal protein is not solely dependent on transmembrane potential. *Infect Immun* 1996; 64: 1070–1074.

141 Koo SP, Yeaman MR, Nast CC, Bayer AS. The cytoplasmic membrane is a primary target for the staphylocidal action of thrombin-induced platelet microbicidal protein. *Infect Immun* 1997; 65: 4795–4800.

142 Yeaman MR, Sullam PM, Dazin PF, Bayer AS. Platelet microbicidal protein alone and in combination with antibiotics reduces *Staphylococcus aureus* adherence to platelets in vitro. *Infect Immun* 1994; 62: 3416–3423.

143 Yeaman MR, Sullam PM, Dazin PF, Ghannoum MA, Edwards JE, Jr., Bayer AS. Fluconazole and platelet microbicidal protein inhibit *Candida* adherence to platelets in vitro. *Antimicrob Agents Chemother* 1994; 38: 1460–1465.

144 Herzberg MC. Oral streptococci in health and disease. In Stevens DL, Kaplan C, eds. *Streptococcal infections*. New York: Oxford University Press; 2000: 333–370.

145 Clawson CC, Rao GH, White JG. Platelet interaction with bacteria. IV. Stimulation of the release reaction. *Am J Pathol* 1975; 81: 411–420.

146 Clawson CC, White JG. Platelet interaction with bacteria. V. Ultrastructure of congenital afibrinogenemic platelets. *Am J Pathol* 1980; 98: 197–211.

147 Hawiger J, Steckley S, Hammond D et al. Staphylococci-induced human platelet injury mediated by protein A and immunoglobulin G Fc fragment receptor. *J Clin Invest* 1979; 64: 931–937.

148 Hawiger J, Kloczewiak M, Timmons S, Strong D, Doolittle RF. Interaction of fibrinogen with staphylococcal clumping factor and with platelets. *Ann NY Acad Sci* 1983; 408: 521–535.

149 McDevitt D, Nanavaty T, House-Pompeo K et al. Characterization of the interaction between the *Staphylococcus aureus* clumping factor (ClfA) and fibrinogen. *Eur J Biochem* 1997; 247: 416–424.

150 Kessler CM, Nussbaum E, Tuazon CU. Disseminated intravascular coagulation associated with *Staphylococcus aureus* septicemia is mediated by peptidoglycan-induced platelet aggregation. *J Infect Dis* 1991; 164: 101–107.

151 Hartleib J, Kohler N, Dickinson RB et al. Protein A is the von Willebrand factor binding protein on *Staphylococcus aureus*. *Blood* 2000; 96: 2149–2156.

152 Herrmann M, Suchard SJ, Boxer LA, Waldvogel FA, Lew PD. Thrombospondin binds to *Staphylococcus aureus* and promotes staphylococcal adherence to surfaces. *Infect Immun* 1991; 59: 279–288.

153 Yamamoto J, Kawano M, Hashimoto M et al. Adjuvant effect of antibodies against von Willebrand Factor, fibrinogen, and fibronectin on staphylokinase-induced thrombolysis as measured using mural thrombi formed in rat mesenteric venules. *Thromb Res* 2000; 97: 327–333.

154 Wang IW, Anderson JM, Marchant RE. S*taphylococcus epidermidis* adhesion to hydrophobic biomedical polymer is mediated by platelets. *J Infect Dis* 1993; 167: 329–336.

155 Bos HM, de Boer RA, Burns GL, Mohammad SF. Evidence that bacteria prefer to adhere to thrombus. *ASAIO J* 1996; 42: M881–M884.

156 Chien Y, Manna AC, Projan SJ, Cheung AL. SarA, a global regulator of virulence determinants in *Staphylococcus aureus*,

Cambridge Books Online © Cambridge University Press, 2009

binds to a conserved motif essential for *sar*-dependent gene regulation. *J Biol Chem* 1999; 274: 37169–37176.

157 Brass LF, Woolkalis MJ, Manning DR. Interactions in platelets between G proteins and the agonists that stimulate phospholipase C and inhibit adenylyl cyclase. *J Biol Chem* 1988; 263: 5348–5355.

158 Fox JE, Reynolds CC, Phillips DR. Calcium-dependent proteolysis occurs during platelet aggregation. *J Biol Chem* 1983; 258: 9973–9981.

159 Pasche B, Ouimet H, Francis S, Loscalzo J. Structural changes in platelet glycoprotein IIb/IIIa by plasmin: determinants and functional consequences. *Blood* 1994; 83: 404–414.

160 Fretto LJ, Fowler WE, McCaslin DR, Erickson HP, McKee PA. Substructure of human von Willebrand factor. Proteolysis by V8 and characterization of two functional domains. *J Biol Chem* 1986; 261: 15679–15689.

161 Jallu V, Pico M, Chevaleyre J, Vezon G, Kunicki TJ, Nurden AT. Characterization of an antibody to the integrin beta 3 subunit (GP IIIa) from a patient with neonatal thrombocytopenia and an inherited deficiency of GP IIb–IIIa complexes in platelets (Glanzmann's thrombasthenia). *Hum Antibodies Hybridomas* 1992; 3: 93–106.

162 Lijnen HR, Van Hoef B, Collen D. Interactions of staphylokinase with human platelets. *Thromb Haemost* 1995; 73: 472–477.

163 Suehiro A, Oura Y, Ueda M, Kakishita E. Inhibitory effect of staphylokinase on platelet aggregation. *Thromb Haemost* 1993; 70: 834–837.

164 Abdelouahed M, Hatmi M, Helft G, Emadi S, Elalamy I, Samama MM. Comparative effects of recombinant staphylokinase and streptokinase on platelet aggregation. *Thromb Haemost* 1997; 77: 815–817.

165 van Zyl WB, Pretorius GH, Hartmann M, Kotze HF. Production of a recombinant antithrombotic and fibrinolytic protein, PLATSAK, in *Escherichia coli*. *Thromb Res* 1997; 88: 419–426.

166 Johnson FB, Donaldson DM. Purification of staphylocidal beta-lysin from rabbit serum. *J Bacteriol* 1968; 96: 589–595.

167 Wu T, Yeaman MR, Bayer AS. In vitro resistance to platelet microbicidal protein correlates with endocarditis source among bacteremic staphylococcal and streptococcal isolates. *Antimicrob Agents Chemother* 1994; 38: 729–732.

168 Mercier RC, Rybak MJ, Bayer AS, Yeaman MR. Influence of platelets and platelet microbicidal protein susceptibility on the fate of *Staphylococcus aureus* in an in vitro model of infective endocarditis. *Infect Immun* 2000; 68: 4699–4705.

169 Usui Y, Ohshima Y, Ichiman Y, Ohtomo T, Suganuma M, Yoshida K. Platelet aggregation induced by strains of various species of coagulase-negative staphylococci. *Microbiol Immunol* 1991; 35: 15–26.

170 Nicolau DP, Marangos MN, Nightingale CH, Quintiliani R. Influence of aspirin on development and treatment of experimental *Staphylococcus aureus* endocarditis. *Antimicrob Agents Chemother* 1995; 39: 1748–1751.

171 Nicolau DP, Tessier PR, Nightingale CH, Quintiliani R. Influence of adjunctive ticlopidine on the treatment of experimental *Staphylococcus aureus* endocarditis. *Int J Antimicrob Agents* 1998; 9: 227–229.

172 Nicolau DP, Tessier PR, Nightingale CH. Beneficial effect of combination antiplatelet therapy on the development of experimental *Staphylococcus aureus* endocarditis. *Int J Antimicrob Agents* 1999; 11: 159–161.

173 Kupferwasser LI, Yeaman MR, Shapiro SM et al. Acetylsalicylic acid reduces vegetation bacterial density, hematogenous bacterial dissemination, and frequency of embolic events in experimental *Staphylococcus aureus* endocarditis through antiplatelet and antibacterial effects. *Circulation* 1999; 99: 2791–2797.

174 Oyen WJ, Boerman OC, Brouwers FM et al. Scintigraphic detection of acute experimental endocarditis with the technetium-99m labelled glycoprotein IIb/IIIa receptor antagonist DMP444. *Eur J Nucl Med* 2000; 27: 392–399.

175 Bhakdi S, Mannhardt U, Muhly M, Hugo F, Ronneberger H, Hungerer KD. Human hyperimmune globulin protects against the cytotoxic action of staphylococcal alpha-toxin in vitro and in vivo. *Infect Immun* 1989; 57: 3214–3220.

176 Bhakdi S, Muhly M, Mannhardt U et al. Staphylococcal alpha toxin promotes blood coagulation via attack on human platelets. *J Exp Med* 1988; 168: 527–542.

177 Jeljaszewicz J, Niewiarowski S, Poplawski A, Blawat L. Platelet aggregation by staphylococcal toxins. *Thromb Diath Haemorrh* 1966; 15: 69–83.

178 Sibelius U, Grandel U, Buerke M et al. Staphylococcal alpha-toxin provokes coronary vasoconstriction and loss in myocardial contractility in perfused rat hearts: role of thromboxane generation. *Circulation* 2000; 101: 78–85.

179 Gareau R, Gruda J, Micusan VV. Effect of toxic shock syndrome toxin-1 on human hemostatic parameters. *Thromb Res* 1989; 54: 349–356.

180 Herzberg MC, MacFarlane GD, Liu P, Erickson PR. The platelet as an inflammatory cell in periodontal diseases. In Genco RJ, ed. *Molecular Pathogenesis of Periodontal Diseases*: Am Soc Microbiol; 1994: 247–255.

180a US Department of Health and Human Services. 'Oral Health in America: a report of the Surgeon General.' Rockville, MD: US Department of Health and Human Services, National Institute of Dental and Craniofacial Research, National Institutes of Health; 2000: 115–120.

181 Fryer RH, Schwobe EP, Woods ML, Rodgers GM. *Chlamydia* species infect human vascular endothelial cells and induce procoagulant activity. *J Investig Med* 1997; 45: 168–174.

182 Zhu J, Quyyumi AA, Norman JE et al. Effects of total pathogen burden on coronary artery disease risk and C-reactive protein levels. *Am J Cardiol* 2000; 85: 140–146.

183 Zhu J, Nieto FJ, Horne BD, Anderson JL, Muhlestein JB, Epstein SE. Prospective study of pathogen burden and risk of myocardial infarction or death. *Circulation* 2001; 103: 45–51.

184 Curtis MA, Macey M, Slaney JM, Howells GL. Platelet activation by Protease I of *Porphyromonas gingivalis* W83. *FEMS Microbiol Lett* 1993; 110: 167–173.

185 Endo Y, Shibazaki M, Nakamura M, Takada H. Contrasting

Cambridge Books Online © Cambridge University Press, 2009

effects of lipopolysaccharides (endotoxins) from oral black-pigmented bacteria and *Enterobacteriaceae* on platelets, a major source of serotonin, and on histamine-forming enzyme in mice. *J Infect Dis* 1997; 175: 1404–1412.
186 Kurpiewski GE, Forrester LJ, Campbell BJ, Barrett JT. Platelet aggregation by *Streptococcus pyogenes*. *Infect Immun* 1983; 39: 704–708.
187 Ryc M, Rotta J. Biological characteristics of peptidoglycans of group A streptococcus and some other bacterial species. II. Immunological mechanisms involved in thrombocytolysis. *J Hyg Epidemiol Microbiol Immunol* 1978; 22: 435–441.
188 Rotta J, Ryc M, Masek K, Zaoral M. Biological activity of synthetic subunits of streptococcus peptidoglycan. I. Pyrogenic and thrombocytolytic activity. *Exp Cell Biol* 1979; 47: 258–268.
189 Akiyama T, Osawa N, Shimanuki K, Yashiro K, Oyake T. Possible role of *Streptococcus pyogenes* in mucocutaneous lymph node syndrome. XII. Variable responses of platelets in MCLS seem to be explainable by streptococcal pyrogenic exotoxin. *Acta Paediatr Jpn* 1991; 33: 20–26.
190 Bernaldo de Quiros JC, Moreno S, Cercenado E et al. Group A streptococcal bacteremia. A 10-year prospective study. *Medicine* (Baltimore) 1997; 76: 238–248.
191 Usui Y, Ohshima Y, Yoshida K. Platelet aggregation by group B streptococci. *J Gen Microbiol* 1987; 133: 1593–1600.
192 Usui Y, Ichiman Y, Ohtomo T, Suganuma M, Yoshida K. Inhibition of platelet aggregation by a whole cell extract from strains of group B streptococcus. *Thromb Res* 1990; 58: 283–291.
193 Wood EG, Gray BM. Type-specific antibody prevents platelet aggregation induced by group B streptococci type III. *J Lab Clin Med* 1986; 107: 322–326.
194 Peevy KJ, Panus P, Longenecker GL, Chartrand SA, Wiseman HJ, Boerth RC et al. Prostaglandin synthetase inhibition in group B streptococcal shock: hematologic and hemodynamic effects. *Pediatr Res* 1986; 20: 864–866.
195 Willcox MD, Oakey HJ, Harty DW, Patrikakis M, Knox KW. Lancefield group C *Streptococcus milleri* group strains aggregate human platelets. *Microb Pathog* 1994; 16: 451–457.
196 Willcox MD. Potential pathogenic properties of members of the '*Streptococcus milleri*' group in relation to the production of endocarditis and abscesses. *J Med Microbiol* 1995; 43: 405–410.
197 Guckian JC. Effect of pneumococci on blood clotting, platelets, and polymorphonuclear leukocytes. *Infect Immun* 1975; 12: 910–918.
198 Zimmerman TS, Spiegelberg HL. Pneumococcus-induced serotonin release from human platelets. Identification of the participating plasma/serum factor as immunoglobulin. *J Clin Invest* 1975; 56: 828–834.
199 Cabellos C, MacIntyre DE, Forrest M, Burroughs M, Prasad S, Tuomanen E. Differing roles for platelet-activating factor during inflammation of the lung and subarachnoid space. The special case of *Streptococcus pneumoniae*. *J Clin Invest* 1992; 90: 612–618.
200 Goldblum SE, Simon TL, Thilsted JP, Babcock TL. Pneumococcus-induced thrombocytopenia in rabbits. *J Lab Clin Med* 1985; 106: 298–307.
201 Kirkpatrick B, Reeves DS, MacGowan AP. A review of the clinical presentation, laboratory features, antimicrobial therapy and outcome of 77 episodes of pneumococcal meningitis occurring in children and adults. *J Infect* 1994; 29: 171–182.
202 Harty DW, Oakey HJ, Patrikakis M, Hume EB, Knox KW. Pathogenic potential of lactobacilli. *Int J Food Microbiol* 1994; 24: 179–189.
203 Kirjavainen PV, Tuomola EM, Crittenden RG et al. In vitro adhesion and platelet aggregation properties of bacteremia-associated lactobacilli. *Infect Immun* 1999; 67: 2653–2655.
204 Czuprynski CJ, Balish E. Interaction of rat platelets with *Listeria monocytogenes*. *Infect Immun* 1981; 33: 103–108.
205 Mahoney NM, Almo SC. Crystallization and preliminary X-ray analysis of human platelet profilin complexed with an oligo proline peptide. *Acta Crystallogr D Biol Crystallogr* 1998; 54: 108–110.
206 Welch MD, Iwamatsu A, Mitchison TJ. Actin polymerization is induced by Arp2/3 protein complex at the surface of *Listeria monocytogenes*. *Nature* 1997; 385: 265–269.
207 Miragliotta G, Lafata M, Jirillo E. Anti-bacterial activity mediated by human platelets. *Agents Actions* 1988; 25: 401–406.
208 Grabarek J, Her GR, Reinhold VN, Hawiger J. Endotoxic lipid A interaction with human platelets. Structure-function analysis of lipid A homologs obtained from *Salmonella minnesota* Re595 lipopolysaccharide. *J Biol Chem* 1990; 265: 8117–8121.
209 Johnson RS, Her GR, Grabarek J, Hawiger J, Reinhold VN. Structural characterization of monophosphoryl lipid A homologs obtained from *Salmonella minnesota* Re595 lipopolysaccharide. *J Biol Chem* 1990; 265: 8108–8116.
210 Timmons S, Huzoor A, Grabarek J, Kloczewiak M, Hawiger J. Mechanism of human platelet activation by endotoxic glycolipid-bearing mutant Re595 of *Salmonella minnesota*. *Blood* 1986; 68: 1015–1023.
211 Wing DA, Yamada T, Hawley HB, Pettit GW. Model for disseminated intravascular coagulation: bacterial sepsis in rhesus monkeys. *J Lab Clin Med* 1978; 92: 239–251.
212 Ulevitch RJ, Cochrane CG, Henson PM, Morrison DC, Doe WF. Mediation systems in bacterial lipopolysaccharide-induced hypotension and disseminated intravascular coagulation. I. The role of complement. *J Exp Med* 1975; 142: 1570–1590.
213 Cooling LL, Walker KE, Gille T, Koerner TA. Shiga toxin binds human platelets via globotriaosylceramide (Pk antigen) and a novel platelet glycosphingolipid. *Infect Immun* 1998; 66: 4355–4366.
214 Keusch GT, Acheson DW. Thrombotic thrombocytopenic purpura associated with Shiga toxins. *Semin Hematol* 1997; 34: 106–116.
215 Kalia N, Jacob S, Brown NJ, Reed MW, Morton D, Bardhan KD. Studies on the gastric mucosal microcirculation. 2. *Helicobacter pylori* water soluble extracts induce platelet aggregation in the gastric mucosal microcirculation in vivo. *Gut* 1997; 41: 748–752.

Cambridge Books Online © Cambridge University Press, 2009

216 Kalia N, Bardhan KD, Reed MW, Jacob S, Brown NJ. Mechanisms of *Helicobacter pylori*-induced rat gastric mucosal microcirculatory disturbances in vivo. *Dig Dis Sci* 2000; 45: 763–772.

217 Cheryk LA, Hooper-McGrevy KE, Gentry PA. Alterations in bovine platelet function and acute phase proteins induced by *Pasteurella haemolytica* A1. *Can J Vet Res* 1998; 62: 1–8.

218 Clinkenbeard KD, Upton ML. Lysis of bovine platelets by *Pasteurella haemolytica* leukotoxin. *Am J Vet Res* 1991; 52: 453–457.

219 Westrop G, Hormozi K, da Costa N, Parton R, Coote J. Structure-function studies of the adenylate cyclase toxin of *Bordetella pertussis* and the leukotoxin of *Pasteurella haemolytica* by heterologous C protein activation and construction of hybrid proteins. *J Bacteriol* 1997; 179: 871–879.

220 Nyarko KA, Coomber BL, Mellors A, Gentry PA. Bovine platelet adhesion is enhanced by leukotoxin and sialoglycoprotease isolated from *Pasteurella haemolytica* A1 cultures. *Vet Microbiol* 1998; 61: 81–91.

221 Konig B, Schonfeld W, Scheffer J, Konig W. Signal transduction in human platelets and inflammatory mediator release induced by genetically cloned hemolysin-positive and -negative *Escherichia coli* strains. *Infect Immun* 1990; 58: 1591–1599.

222 Taneyama C, Sasao J, Senna S et al. Protective effects of ONO 3708, a new thromboxane A2 receptor antagonist, during experimental endotoxin shock. *Circ Shock* 1989; 28: 69–77.

223 Sheu JR, Hung WC, Su CH et al. The antiplatelet activity of *Escherichia coli* lipopolysaccharide is mediated through a nitric oxide/cyclic GMP pathway. *Eur J Haematol* 1999; 62: 317–326.

224 Csako G, Suba EA, Elin RJ. Endotoxin-induced platelet activation in human whole blood in vitro. *Thromb Haemost* 1988; 59: 378–382.

225 Morrison DC, Kline LF, Oades ZG, Henson PM. Mechanisms of lipopolysaccharide-initiated rabbit platelet responses: alternative complement pathway dependence of the lytic response. *Infect Immun* 1978; 20: 7447–51.

226 Shibazaki M, Nakamura M, Endo Y. Biphasic, organ-specific, and strain-specific accumulation of platelets induced in mice by a lipopolysaccharide from *Escherichia coli* and its possible involvement in shock. *Infect Immun* 1996; 64: 5290–5294.

227 Jourdain M, Tournoys A, Leroy X et al. Effects of *N* omega-nitro-L-arginine methyl ester on the endotoxin-induced disseminated intravascular coagulation in porcine septic shock. *Crit Care Med* 1997; 25: 452–459.

228 Galbe JL, Guy E, Zapatero JM, Peerschke EI, Benach JL. Vascular clearance of *Borrelia burgdorferi* in rats. *Microb Pathog* 1993; 14: 187–201.

229 Coburn J, Barthold SW, Leong JM. Diverse Lyme disease spirochetes bind integrin alpha IIb beta 3 on human platelets. *Infect Immun* 1994; 62: 5559–5567.

230 Ntchobo H, Rothermel H, Chege W, Steere AC, Coburn J. Recognition of multiple antibody epitopes throughout *Borrelia burgdorferi* p66, a candidate adhesin, in patients with early or late manifestations of Lyme disease. *Infect Immun* 2001; 69: 1953–1956.

231 Dalldorf FG, Jennette JC. Fatal meningococcal septicemia. *Arch Pathol Lab Med* 1977; 101: 6–9.

232 Vik-Mo H, Lote K, Nordoy A. Disseminated intravascular coagulation in patients with meningococcal infection: laboratory diagnosis and prognostic factors. *Scand J Infect Dis* 1978; 10: 187–191.

233 Jolivet-Reynaud C, Launay JM, Alouf JE. Damaging effects of *Clostridium perfringens* delta toxin on blood platelets and their relevance to ganglioside GM2. *Arch Biochem Biophys* 1988; 262: 59–66.

234 Akbar H, Navran SS, Chang J, Miller DD, Feller DR. Investigation of the effects of phospholipase C on human platelets: evidence that aggregation induced by phospholipase C is independent of prostaglandin generation, released ADP and is modulated by cyclic AMP. *Thromb Res* 1982; 27: 405–417.

235 Navran SS, Romstedt K, Chang J, Miller DD, Feller DR, Huzoor A. Human platelet activation by bacterial phospholipase C is mediated by phosphatidylinositol hydrolysis but not generation of phosphatidic acid: inhibition by a selective inhibitor of phospholipase C. *Thromb Res* 1984; 33: 499–510.

236 Bryant AE, Chen RY, Nagata Y et al. Clostridial gas gangrene. II. Phospholipase C-induced activation of platelet gpIIbIIIa mediates vascular occlusion and myonecrosis in *Clostridium perfringens* gas gangrene. *J Infect Dis* 2000; 182: 808–815.

237 Ohsaka A, Tsuchiya M, Oshio C, Miyairi M, Suzuki K, Yamakawa Y. Aggregation of platelets in the mesenteric microcirculation of the rat induced by alpha-toxin (phospholipase C) of *Clostridium perfringens*. *Toxicon* 1978; 16: 333–341.

238 Mauco G, Chap H, Simon MF, Douste-Blazy L. Phosphatidic and lysophosphatidic acid production in phospholipase C- and thrombin-treated platelets. Possible involvement of a platelet lipase. *Biochimie* 1978; 60: 653–661.

239 Chap H, Perret B, Mauco G et al. Organization and role of platelet membrane phospholipids as studied with phospholipases A2 from various venoms and phospholipases C from bacterial origin. *Toxicon* 1982; 20: 291–298.

240 Aktories K, Just I, Rosenthal W. Different types of ADP-ribose protein bonds formed by botulinum C2 toxin, botulinum ADP-ribosyltransferase C3 and pertussis toxin. *Biochem Biophys Res Commun* 1988; 156: 361–367.

241 Nemoto Y, Namba T, Teru-uchi T, Ushikubi F, Morii N, Narumiya S. A rho gene product in human blood platelets. I. Identification of the platelet substrate for botulinum C3 ADP-ribosyltransferase as rhoA protein. *J Biol Chem* 1992; 267: 20916–20920.

242 Morii N, Teru-uchi T, Tominaga T et al. A rho gene product in human blood platelets. II. Effects of the ADP-ribosylation by botulinum C3 ADP-ribosyltransferase on platelet aggregation. *J Biol Chem* 1992; 267: 20921–20926.

Cambridge Books Online © Cambridge University Press, 2009

243 Leng L, Kashiwagi H, Ren XD, Shattil SJ. RhoA and the function of platelet integrin alphaIIbbeta3. *Blood* 1998; 91: 4206–4215.

244 Klages B, Brandt U, Simon MI, Schultz G, Offermanns S. Activation of G12/G13 results in shape change and Rho/Rho-kinase-mediated myosin light chain phosphorylation in mouse platelets. *J Cell Biol* 1999; 144: 745–754.

245 Retzer M, Siess W, Essler M. Mildly oxidised low density lipoprotein induces platelet shape change via Rho-kinase-dependent phosphorylation of myosin light chain and moesin. *FEBS Lett* 2000; 466: 70–74.

246 Kurose I, Pothoulakis C, LaMont JT et al. *Clostridium difficile* toxin A-induced microvascular dysfunction. Role of histamine. *J Clin Invest* 1994; 94: 1919–1926.

247 Permin H, Skov PS, Norn S, Hoiby N, Schiotz PO. Platelet ^{3}H-serotonin releasing immune complexes induced by *Pseudomonas aeruginosa* in cystic fibrosis. *Allergy* 1982; 37: 93–100.

248 Konig B, Jaeger KE, Konig W. Induction of inflammatory mediator release (12-hydroxyeicosatetraenoic acid) from human platelets by *Pseudomonas aeruginosa. Int Arch Allergy Immunol* 1994; 104: 33–41.

249 Konig B, Jaeger KE, Sage AE, Vasil ML, Konig W. Role of *Pseudomonas aeruginosa* lipase in inflammatory mediator release from human inflammatory effector cells (platelets, granulocytes, and monocytes. *Infect Immun* 1996; 64: 3252–3258.

250 Myrvold HE, Brandberg A. Microembolism in experimental septic shock. Distribution of platelets and fibrinogen after intravenous injection of disintegrated *Pseudomonas* bacteria to dogs. *Eur Surg Res* 1977; 9: 34–47.

251 Bayer AS, Ramos MD, Menzies BE, Yeaman MR, Shen AJ, Cheung AL. Hyperproduction of alpha-toxin by *Staphylococcus aureus* results in paradoxically reduced virulence in experimental endocarditis: a host defense role for platelet microbicidal proteins. *Infect Immun* 1997; 65: 4652–4660.

252 Ninomiya M, Matsushita O, Minami J, Sakamoto H, Nakano M, Okabe A. Role of alpha-toxin in *Clostridium perfringens* infection determined by using recombinants of *C. perfringens* and *Bacillus subtilis. Infect Immun* 1994; 62: 5032–5039.

253 Just I, Schallehn G, Aktories K. ADP-ribosylation of small GTP-binding proteins by *Bacillus cereus. Biochem Biophys Res Commun* 1992; 183: 931–936.

254 Dhar A, Shukla SD. Release of a membrane surface glycoprotein from human platelets by phosphatidylinositol specific phospholipase(s) C. *Biochim Biophys Acta* 1991; 1091: 15–21.

255 Faung ST, Chiu L, Wang CT. Platelet lysis and functional perturbation by 13-methyl myristate. The major fatty acid in *Flavobacterium ranacida. Thromb Res* 1996; 81: 91–100.

256 Kamiyama T, Umino T, Satoh T et al. Sulfobacins A and B, novel von Willebrand factor receptor antagonists. I. Production, isolation, characterization and biological activities. *J Antibiot (Tokyo)* 1995; 48: 924–928.

257 Forrester LJ, Campbell BJ, Berg JN, Barrett JT. Aggregation of platelets by *Fusobacterium necrophorum. J Clin Microbiol* 1985; 22: 245–249.

258 Kanoe M, Yamanaka M. Bovine platelet aggregation by *Fusobacterium necrophorum. J Med Microbiol* 1989; 29: 13–17.

259 Horose M, Kiyoyama H, Ogawa H, Shinjo T. Aggregation of bovine platelets by *Fusobacterium necrophorum. Vet Microbiol* 1992; 32: 343–350.

260 Cooper HA, Bennett WP, Kreger A, Lyerly D, Wagner RH. Proteolysis of human fixed, washed platelets by gram-negative bacterial metalloproteases: effect on von Willebrand factor-human platelet interactions. *Ann NY Acad Sci* 1981; 370: 179–190.

261 Cooper HA, Bennett WP, Kreger A, Lyerly D, Wagner RH. The effect of extracellular proteases from gram-negative bacteria on the interaction of von Willebrand factor with human platelets. *J Lab Clin Med* 1981; 97: 379–389.

262 Cooper HA, Bennett WP, White GC, 2nd, Wagner RH. Hydrolysis of human platelet membrane glycoproteins with a *Serratia marcescens* metalloprotease: effect on response to thrombin and von Willebrand factor. *Proc Natl Acad Sci USA* 1982; 79: 1433–1437.

263 Kinlough-Rathbone RL, Perry DW, Packham MA. Contrasting effects of thrombin and the thrombin receptor peptide, SFLLRN, on aggregation and release of 14C-serotonin by human platelets pretreated with chymotrypsin or *Serratia marcescens* protease. *Thromb Haemost* 1995; 73: 122–125.

264 McGowan EB, Detwiler TC. Differential effect of *Serratia protease* on platelet surface glycoproteins Ib and V. *Blood* 1985; 65: 1033–1035.

265 Dickneite G, Czech J. Combination of antibiotic treatment with the thrombin inhibitor recombinant hirudin for the therapy of experimental *Klebsiella pneumoniae* sepsis. *Thromb Haemost* 1994; 71: 768–772.

266 Dickneite G, Czech J, Keuper H. Formation of fibrin monomers in experimental disseminated intravascular coagulation and its inhibition by recombinant hirudin. *Circ Shock* 1994; 42: 183–189.

267 Iwaki M, Kamachi K, Heveker N, Konda T. Suppression of platelet aggregation by *Bordetella pertussis* adenylate cyclase toxin. *Infect Immun* 1999; 67: 2763–2768.

268 Reisner BS, Straley SC. *Yersinia pestis* YopM: thrombin binding and overexpression. *Infect Immun* 1992; 60: 5242–5252.

269 Simonet M, Triadou P, Frehel C, Morel-Kopp MC, Kaplan C, Berche P. Human platelet aggregation by *Yersinia pseudotuberculosis* is mediated by invasin. *Infect Immun* 1992; 60: 366–373.

270 Isogai E, Kitagawa H, Isogai H et al. Effects of leptospiral lipopolysaccharide on rabbit platelets. *Zentralbl Bakteriol* 1989; 271: 186–196.

271 Isogai E, Hirose K, Kimura K et al. Role of platelet-activating-

Cambridge Books Online © Cambridge University Press, 2009

factor (PAF) on cellular responses after stimulation with leptospire lipopolysaccharide. *Microbiol Immunol* 1997; 41: 271–275.
272 Nicodemo AC, Duarte MI, Alves VA, Takakura CF, Santos RT, Nicodemo EL. Lung lesions in human leptospirosis: microscopic, immunohistochemical, and ultrastructural features related to thrombocytopenia. *Am J Trop Med Hyg* 1997; 56: 181–187.
273 Sibazaki M, Kawabata Y, Yokochi T, Nishida A, Takada H, Endo Y. Complement-dependent accumulation and degradation of platelets in the lung and liver induced by injection of lipopolysaccharides. *Infec Immun* 1999; 67: 5186–5191.

Cambridge Books Online © Cambridge University Press, 2009

Interactions of viruses and platelets and the inactivation of viruses in platelet concentrates prepared for transfusion

Laurence Corash

Cerus Corporation and Department of Laboratory Medicine, University of California, San Francisco, USA

Introduction

Viruses have long been causally linked, theoretically, with acquired idiopathic thrombocytopenia[1] and more recently have been associated directly with megakaryocytes and platelets during retroviral infections caused by the human immunodeficiency virus and human T-cell lymphotrophic viruses[2]. While the pathophysiology of the association of these viruses with megakaryocytes and platelets remains incompletely understood, recent investigations with molecular methods have begun to elucidate some potential mechanisms of the complex interactions between viruses and platelets. This chapter will focus on the interactions of viruses and platelets that are associated with human platelet disorders. In addition, recent technology developed to inactivate viruses in platelet concentrates prepared for transfusion is of interest. This technology appears to be an important intervention to further improve the safety of transfusion, and also may well be a significant modality for prevention of platelet transfusion associated infections due to new viruses that may enter the donor population in the future[3].

Substantial improvement in the safety of platelet transfusion has been achieved through addition of new tests, such as nucleic acid tests, yet residual risk persists in association with transfusion of platelet concentrates[4]. Transfusion of platelet concentrates has been implicated in the transmission of viruses, bacteria, and protozoa[5]. While it is commonly recognized that hepatitis B virus (HBV), hepatitis C virus (HCV), cytomegalovirus (CMV), and the retroviruses, such as human immunodeficiency virus (HIV) and the human lymphotrophic viruses (HTLV) can be transmitted through cellular components, other viruses are emerging as potentially significant transfusion-associated infectious agents[3]. More importantly, new infectious agents may periodically enter the donor population before they can be definitively identified and tested for to maintain consistent safety of the blood supply. The paradigm for this possibility is the HIV pandemic, which erupted in 1979. Another approach to improving the safety of platelet transfusion is the use of methods to inactivate potential contaminating viruses[6]. In order to develop effective viral inactivation methods, it is necessary to understand the interactions of viruses with platelets and to demonstrate that viruses can be inactivated in various platelet subcellular compartments. Since 1990 several methods to inactivate infectious pathogens in platelet concentrates have been developed and have entered the advanced clinical trial phase.

Interactions of viruses, platelets and megakaryocytes

Childhood acquired thrombocytopenia has long been associated with antecedent viral infections. Evidence based on viral isolates during the acute phase of these infections has been reported for infections due to varicella zoster virus, rubella virus, Epstein–Barr virus, influenza, and the human immunodeficiency virus type 1[1]. Several pathophysiological mechanisms have been postulated to explain the interactions of viruses with platelets in these frequently transient disorders. The most commonly asserted mechanism of action is the increased clearance of platelets by antibody directed against platelet glycoproteins and by circulating immune complexes potentially bound to platelet Fc receptors[7,8]. For example, the immunologic interactions induced by HIV-1 infection are

complex based on evidence demonstrating antibody directed against a platelet GPIIIa peptide (49–66); and purification of circulating immune complexes revealed the presence of an antiidiotype IgM which was a blocking antibody directed against the anti-GPIIIa IgG[8]. Thus, infection with HIV-1 appeared to give rise to multifactoral autoimmune responses.

More recently, evidence has been presented to suggest that certain viruses may directly infect megakaryocytes and thus interfere with thrombopoiesis by unknown mechanisms. Zucker-Franklin et al. have shown that murine and human megakaryocytes cocultured with retrovirus infected cells resulted in the presence of virus, by ultrastructural analysis, in megakaryocytes and platelets[9]. These observations are consistent with earlier reports that after experimental infection with murine retrovirus, virus was found in megakaryocytes[10,11]. At least two laboratories have reported the presence of HIV nucleic acid sequences in megakaryocytes of infected patients[12,13]. Some workers have postulated that infection of megakaryocytes, perhaps mediated by CD4 molecules expressed by megakaryocytes, results in suppressed thrombopoiesis[13]. Subsequently, Zucker-Franklin et al. suggested that the prevalence of other human retroviruses in humans may be greater than expected when sensitive molecular methods are used[2].

Additional evidence regarding the complex interactions of viruses with platelets has come from studies of HIV type 1 virus obtained from human infections. Lee and coworkers reported that platelets isolated from whole blood donations collected from HIV-1 positive asymptomatic donors contained high levels of HIV RNA after extensive washing and storage of platelets at both 4 °C and 22 °C[14]. While the washed platelets containing HIV-1 RNA did not demonstrate infectivity, platelets incubated in vitro with cell-free virus showed strong attachment and retained infectivity by in vitro assays. In combination with evidence that platelet concentrates from recognized HIV infected donors have transmitted HIV infections, and a recent report that platelets obtained from a donor prior to serologic conversion transmitted HIV infection, there is little doubt that HIV associated with platelets is infectious[15]. Moreover, the demonstration that the viral load in this implicated donor contained less than 40 copies of HIV per ml suggested that minipool nucleic acid-based detection strategies may not be sufficient to detect early stage infected blood donors[15].

In addition to HIV, other experimental evidence suggests a more generalized association of pathogenic viruses with platelets and megakaryocytes. For example, early work with murine cytomegalovirus infections (CMV) indicated that this virus exerts a direct suppressive effect on thrombopoiesis of megakaryocytes[16]. Others have shown evidence of direct infection by CMV of hematopoietic progenitor cells[17].

Hantavirus infections are associated with acute severe thrombocytopenia and hemorrhagic clinical syndromes. Based on recent studies, hantaviruses have been postulated to regulate β3-integrins which are critical adhesive and activation receptors on platelets[18]. These potential interactions may explain some of the pathophysiology due to hantavirus infection. The β3-specific integrins appear to mediate entry of hantavirus into endothelial cells leading to development of the hemorrhagic fever with renal syndrome (HFRS) and the hantavirus pulmonary syndrome (HPS). Cells lacking the β3-integrins are not permissive for hantavirus infection, and antibodies to the β3-integrins block experimental hantavirus infection. The absence or abnormality of the platelet β3 integrin complex GPIIb/IIIa is associated with a hemorrhagic syndrome, Glanzmann's thrombasthenia. The potential binding of hantaviruses to GPIIb/IIIa on platelets could interfere with platelet adhesion or potentiate platelet activation facilitating increased clearance of platelets or microvascular thrombotic events.

Bovine diarrhea virus (BVDV), a flavivirus frequently used as a model for hepatitis C virus (HCV), has been shown to be present in the platelets of experimentally infected calves[19]. This interaction of BVDV and bovine platelets may explain the hemorrhagic syndrome associated with this infection. More direct evidence of HCV interactions with platelets has come from molecular assays for detection of HCV nucleic sequences in platelets and megakaryocytes[20]. Thrombocytopenia is a frequent, although rarely severe, complication of HCV infection. Platelets from patients with HCV infection have been shown to contain HCV RNA sequences[21,22]. In further studies, Li et al. incubated serum from an HCV infected patient with a megakaryoblastic cell line derived from a patient with chronic myelogenous leukemia. The megakaryoblasts were positive for HCV RNA and minus strand HCV RNA, indicative of active infection[20]. Furthermore, HCV proteins, including core, envelope, and nonstructural proteins NS3 and NS4 were identified in cell cultures by Western blotting, and virus particles were identified by electron microscopy in megakaryoblasts. These studies clearly indicate that HCV can infect and replicate in megakaryoblast cell lines and are consistent with demonstration of HCV nucleic acid sequences in the platelets of patients infected with HCV.

Parvovirus B-19 is well recognized to be associated with pure red cell aplasia and is not commonly associated with thrombocytopenia. Patients with combined anemia and thrombocytopenia have been reported, and one patient with both HCV and parvovirus B-19 documented infection

Cambridge Books Online © Cambridge University Press, 2009

has been described with amegakaryocytosis which resolved after resolution of the parvovirus B-19 infection[23]. However, it remains unclear whether megakaryocytes or platelets were directly infected with the virus as documented for erythroblasts.

Platelet transfusion associated viral infections

Currently, prevention of transfusion-associated viral disease depends upon predonation evaluation of potential donors followed by serologic testing for infectious pathogens including: human immunodeficiency virus (HIV-1 and -2), human T-cell lymphotrophic viruses (HTLV-I), hepatitis B virus (HBV), and hepatitis C virus (HCV). Cytomegalovirus (CMV) screening is generally performed after blood collection, when CMV seronegative products are required. In addition to these agents, blood is tested for the syphilis pathogen (*Treponema pallidum*). Testing is not routinely done for parvovirus B19, hepatitis A virus (HAV), hepatitis G virus (HGV), hepatitis E virus (HEV), human herpes viruses (HHV-6 and HHV-8), Epstein–Barr virus, bacteria, or protozoa. Although continuing improvements in testing have greatly reduced the transmission of viral disease by labile blood components, viruses may still be transmitted because diagnostic tests may be insensitive during the 'window period' before sero-conversion[15]. Even direct tests for a virus, such as the hepatitis B surface antigen test, have a sensitivity threshold that allows contaminated components to escape detection. Recently, a case of HCV transfusion-associated transmission was documented despite single-sample nucleic acid testing with highly sensitive methods[24]. Similarly, a case of HIV transfusion-associated infection was reported during the window period with low virus copy numbers not detected by nucleic acid testing under conditions of minipool dilution[15].

Prior to introduction of nucleic acid testing, estimates of the frequency of viral transmission due to transfusion of blood components, per donor, were 1 in 100 000 for HCV, 1 in 63 000 for HBV, 1 in 680 000 for HIV, and 1 in 641 000 for HTLV-1[4,25]. The aggregate risk of receiving a blood component contaminated with one of the viruses for which sensitive tests are in place has been estimated to be 1 in 34 000[26]. A recent US government report estimated that the average transfusion episode results in exposure to five donors[27]. Thus, for a transfusion episode, the risk of receiving a component contaminated with virus may be as high as 1 in 6800.

Bacterial contamination of platelet concentrates is a persistent problem due to room temperature (20 °C–24 °C) storage for up to 5 days prior to use. Bacterial contamination may come directly from the donor or from an external source. Unlike viruses, a small number of contaminating bacteria can replicate to $>10^7$ per ml after 5 days of storage. A wide variety of bacteria have been cultured from patients with transfusion-transmitted septicemia[28,29]. Although the number of reported cases of serious transfusion-transmitted sepsis is small, there are no routine laboratory tests to detect bacterial contamination of platelet units. Estimates of the frequency of bacterial contamination range up to 0.4% per platelet concentrate[30]. A 1991 survey by Morrow and coworkers identified bacterial contamination culminating in a septic response from 6 of 10 219 transfusions of pooled random donor platelets, a frequency of approximately 1 in 1700[29]. A prospective study of 3584 platelet transfusions in 161 bone marrow transplant patients demonstrated the risk of symptomatic bacteremia as 1 per 16 patients, 1 per 350 transfusions, and 1 per 2100 platelet units[31]. These frequencies are significant considering that over 8 million units of platelet concentrates are transfused annually in the United States alone[32].

The logistics and costs of continued expansion of testing processes, for example nucleic acid testing, have been questioned[25]. Testing remains a reactive strategy to insure blood component safety, since new pathogens may enter the donor population before adequate tests can be implemented. Moreover, the sensitivity of all testing methods is limited inherently by the volume of blood that can be analysed. A complementary approach to improving the safety of blood component transfusion is inactivation of infectious pathogens in blood components by using a process that treats the entire blood component. For example, treatment of plasma fractions with the solvent detergent process has demonstrated the benefits of this approach[33]. A robust inactivation technology that is compatible with current blood component processing procedures offers the potential for improving transfusion safety beyond that achievable by testing. Moreover, a nucleic acid targeted technology capable of inactivating residual leukocytes may confer additional benefits due to inhibition of cytokine synthesis, lymphocyte proliferation, and antigen presentation. Donor leukocytes are associated with a variety of adverse immune events ranging in severity from febrile transfusion reactions to alloimmunization and graft versus host disease[34,35]. Although a number of measures have been implemented to reduce the likelihood of these adverse immune reactions, a robust nucleic acid targeted pathogen inactivation process offers the potential to inactivate all leukocytes, as well as infectious pathogens. Over the past decade a number of laboratories have reported investigations to apply pathogen inactivation technology

Cambridge Books Online © Cambridge University Press, 2009

Table 52.1. Clinical trials of methods for inactivation of pathogens in platelet concentrates

Component	System	Design	Subjects	Primary endpoint	Phase
Single donor	S-59	Auto/5-day old[c]	23	Viability	Ia
Single donor	S-59	Auto/5-day old	10	S-59 Kinetics	Ib
Single donor	S-59	Auto/5 day old	16	Viability + CAD[b]	IIa
Single donor	S-59	Auto/5 day old	15	Viability + CAD + 2500 cGy	IIb
Single donor	S-59	Allogeneic[d]	42	Hemostasis	IIc
Buffy coat	S-59	Allogeneic[d]	103	Count increments	III
Single donor[a]	S-59	Allogeneic[d]	671	Bleeding	III

Notes:
[a] Phase I, II, and III trials of S-59 platelets have been completed and are discussed in more detail in the text.
[b] CAD, compound adsorption device for reduction of S-59 levels postillumination.
[c] Autologous donor platelets stored for 5 days before transfusion.
[d] Allogeneic donor platelets stored for up to 5 days before transfusion.

to platelet concentrates. These efforts have focused on several methods, one of which is now in advanced clinical trials (Table 52.1).

Systems for inactivation of viruses in platelet concentrates

Considerable effort has been devoted to investigations to develop methods for pathogen inactivation in platelet concentrates (Table 52.2, Table 52.3). The potential processes can be divided into two basic groups: psoralens and photodynamic methods. The psoralen-mediated processes generally utilize nucleic acid targeted adduct formation, while the photodynamic processes rely on the production of active oxygen species as the primary mechanism for pathogen inactivation. The photodynamic methods generally do not provide sufficient pathogen inactivation and are associated with unacceptable levels of platelet injury[36]. Psoralen methods have been more extensively investigated and more progress has been made with psoralens than with the other systems.

Early investigations with psoralen mediated pathogen inactivation were conducted with 8-methxyopsoralen (8–MOP) based on the history of prior human use to treat psoriasis and cutaneous T cell lymphoma[37,38]. These initial studies by Lin and coworkers established the principle of psoralen-mediated pathogen inactivation, but 8-MOP photochemical treatment was not a sufficiently rapid process for treatment of platelet concentrates in clinical use[39,40].

Several laboratories have investigated the use of AMT, a synthetic psoralen with enhanced nucleic acid binding

Table 52.2. Psoralen methods used to inactivate infectious pathogens and leukocytes in platelet concentrates

Photoreactive agent	Target	Reference
8-MOP	fd, R17, FeLV, E. coli *S. aureus*	40
8-MOP	MCMV, FeRTV	55
8-MOP	HIV	56
8-MOP	DHBV	50
8-MOP	12 pathogenic bacteria	57
AMT	VSV	41
AMT	HIV	42,58
AMT	VSV, Sindbis	59
PSR-Br	Bacteriophage	60,61
S-59	Pathogenic bacteria	45
S-59	Leukocytes	46
S-59	HIV, DHBV, BVDV CMV, bacteria	45
S-59	HIV, bacteria	47

Note:
fd – bacteriophage, R17 – bacteriophage, MCMV – murine cytomegalovirus, FeRTV – feline rhinotracheitis virus, HIV – human immunodeficiency virus, HSV – herpes simplex virus, CMV – cytomegalovirus, VSV – vesicular stomatitis virus, FeLV – feline leukemia virus, Sinbis – Sindbis virus, 8-MOP – 8-methoxypsoralen, AMT – aminomethyltrimethylpsoralen, PSR-Br – brominated psoralens.

Cambridge Books Online © Cambridge University Press, 2009

Table 52.3. Photodynamic methods used to inactivate infectious pathogens in platelet concentrates

Photoreactive agent	Target	Reference
UVB	Poliovirus	62
Merocyanine 540	VSV	63
Merocyanine 540	HSV, MS2, F6	41
Methylene blue	Unspecified	64
Phthalocyanines	VSV	65
Riboflavin[a]	HIV, BVDV, PRV, PPV, HSV Vaccinia, Bacteria	66

Note:
VSV – vesicular stomatitis virus, MS2 – bacteriophage, F6 – bacteriophage, HSV – herpes simplex virus, HIV – human immunodeficiency virus, UVB – ultraviolet B light (280–320 nm), BVDV– bovine diarrhea virus, PRV – pseudorabies virus, PPV – porcine parvovirus, HSV – herpes simplex virus.
[a] Studies conducted in plasma: media mixture (90 : 10) without platelets.

efficiency[41,42]. While AMT has increased nucleic acid binding affinity compared to 8–MOP, it exhibits mutagenicity in the absence of light, and thus has an unfavourable toxicology profile. Several classes of new psoralens have been synthesized which offer potential advantages over AMT and 8–MOP. The halogenated psoralens do not appear to be sufficiently effective for viral inactivation and in preliminary studies demonstrate adverse effects on platelet viability[43,44].

A new class of amino psoralens (Helinx Technology) has been synthesized and shown to be highly effective for inactivation of pathogenic viruses, bacteria and leukocytes in platelet concentrates during a 3-minute UVA illumination[45,46]. An integrated system (INTERCEPT Platelet System) using the lead compound, S-59, has been developed for large-scale production of platelet concentrates. This device consists of a series of interconnected plastic containers in a closed system for preparation of therapeutic doses of single donor and pooled platelets. The system contains an integral compound absorption device (CAD) to lower the post-treatment levels of residual S-59 and free S-59 photoproducts. In addition to inactivation of HIV and bacteria[45,47], Lin and coworkers demonstrated that human platelet concentrates (300 ml) contaminated with high titres of HCV ($10^{4.5}$) and HBV ($10^{5.5}$) treated with the S-59 process did not transmit hepatitis after transfusion into naïve chimpanzees[48]. Importantly, both cell-free and cell-associated viruses were inactivated, including duck hepatitis B virus (DHBV) which is a stringent model for human hepatitis B virus[49,50]. Importantly, the complete inactivation of bacteria, which if viable can proliferate during platelet storage, is a rigorous demonstration that the entire platelet product has been treated. Other studies confirmed that these novel psoralens inactivated high levels of T cells, inhibited leukocyte cytokine synthesis during platelet storage, and inhibited nucleic acid amplification of small nucleic acid sequences[46]. More importantly, treatment of T cells with the S-59 process prevented transfusion-associated graft versus host disease in both immune competent and immune compromised models of murine stem cell transplantation[46].

Helinx technology with the novel psoralen, S-59, has undergone extensive clinical study[51]. Phase I and II studies using Helinx-treated, 5 day-old platelets transfused in healthy subjects have shown adequate viability. In these studies, photochemically treated platelets were well tolerated during and after transfusion of full doses (300 ml, 3.0×10^{11} platelets). The therapeutic efficacy of Helinx-treated platelets was examined in a pilot study of profoundly thrombocytopenic patients. This clinical trial was designed to evaluate the hemostatic efficacy of S-59 treated platelet concentrates. In this study, transfusion of Helinx-treated platelet concentrates resulted in shortening of markedly prolonged cutaneous template bleeding times, and provided adequate platelet count increments and acceptable intervals to the next platelet transfusion[52]. The S-59 platelet concentrates were well tolerated.

Two randomized, controlled, blinded, clinical trials to determine the therapeutic efficacy of multiple transfusions of S-59 treated platelet concentrates have been completed. A European trial (*EuroSPRITE*) using pooled random donor platelets prepared by the buffy coat method enrolled 106 thrombocytopenic patients to receive up to 8 weeks of platelet transfusion support with either Helinx treated or standard platelet concentrates. One hundred and three patients were transfused during this study. The primary endpoint was the platelet count increment 1 hour after transfusion. Secondary endpoints included the platelet count increment 24 hours after transfusion, clinical hemostasis, refractoriness to transfusion, the intertransfusion interval and the frequency of acute transfusion reactions. This study demonstrated that platelet pools treated with Helinx technology and stored for up to 5 days provided count increments comparable to those of platelet pools not treated with pathogen inactivation[53]. Furthermore, Helinx treated platelets were comparable to untreated platelets with respect to transfusion intervals, refractoriness to platelet transfusion, the proportion of patients with major hemorrhage and safety.

Cambridge Books Online © Cambridge University Press, 2009

A larger US study (*SPRINT*) enrolled 671 thrombocytopenic patients to receive up to 4 weeks of platelet transfusion support with either Helinx treated platelets or standard single donor platelets. The primary endpoint in the US trial is the proportion of patients with grade 2 bleeding during the period of platelet support[54]. The secondary endpoints include the proportion of patients with high grade bleeding, platelet count increments and the same spectrum of endpoints as in the European study. This study will be unblinded later this year.

Conclusions

Viruses exhibit complex interactions with both megakaryocytes and platelets, and cause abnormalities of both platelet function and thrombopoiesis. These interactions occur by indirect immunologic mechanisms as a consequence of viral infection and via direct infection of megakaryocytes, and perhaps platelets. As a result of these interactions, these viruses give rise to hemorrhagic syndromes, and these viruses can be transmitted by transfusion of platelets. Considerable progress has been made in the development of technologies to inactivate infectious pathogens in platelet concentrates. These technologies have entered advanced clinical trials, and one platelet system has completed Phase III studies in Europe and the United States. These systems for inactivation of viruses in platelet products have the potential to markedly change the way in which platelet components are prepared and to further improve the safety of transfusion support of patients.

References

1 Rand ML, Wright JF. Virus-associated idiopathic thrombocytopenia purpura. *Transfus Sci* 1998; 19: 253–259.
2 Zucker-Franklin D, Pancake BA, Marmor M, Legler PM. Reexamination of human T cell lymphotrophic virus (HTLV-I/II) prevalence. *Proc Natl Acad Sci USA* 1997; 94: 6403–6407.
3 Allain JP. Emerging viruses in blood transfusion. *Vox Sang* 2000; 78 (Suppl 2): 243–248.
4 Glynn SA, Kleinman SH, Schreiber GB et al. Trends in incidence and prevalence of major transfusion-transmissible viral infections in US blood donors, 1991 to 1996. *JAMA* 2000; 284: 229–235.
5 Dodd RY. Will blood products be free of infectious agents? In Nance SJ, ed. *Transfusion Medicine in the 1990's.* Arlington,VA: American Association of Blood Banks; 1990: 223–251.
6 Corash L. Inactivation of viruses, bacteria, protozoa, and leukocytes in platelet and red cell concentrates. *Vox Sang* 2000; 78(Suppl 2): 205–210.
7 Walsh CM, Nardi MA, Karpatkin S. On the mechanism of thrombocytopenic purpura in sexually-active homosexual men. *N Engl J Med* 1984; 311: 635–639.
8 Nardi M, Karpatkin S. Antiidiotype antibody against platelet anti-GPIIIa contributes to the regulation of thrombocytopenia in HIV-1–ITP patients. *J Exp Med* 2000; 191: 2093–2100.
9 Zucker-Franklin D, Seremetis S, Zheng ZY. Internalization of human immunodeficiency virus type I and other retroviruses by megakaryocytes and platelets. *Blood* 1990; 75: 1920–1923.
10 DeHarven E, Friend C. Further electron microscopic studies of a mouse leukemia induced by cell-free filtrates. *J Biophys Biochem Cytol* 1960; 7: 747.
11 Brown WM, Axelrad AA. Effect of Friend leukemia virus on megakaryocytes and platelets in mice. *Int J Cancer* 1976; 18: 764.
12 Zucker-Franklin D, Cao Y. Megakaryocytes of human immunodeficiency virus-infected individuals express viral RNA. *Proc Natl Acad Sci USA* 1989; 86: 5595.
13 Louache F, Bettaieb A, Henri A et al. Infection of megakaryocytes by human immunodeficiency virus in seropositive patients with immune thrombocytopenia. *Blood* 1991; 78: 1697–1705.
14 Lee TH, Stromberg RR, Heitman JW, Sawyer L, Hanson CV, Busch MP. Distribution of HIV type 1 (HIV-1) in blood components: detection and significance of high levels of HIV-1 associated with platelets. *Transfusion* 1998; 38: 580–588.
15 Ling AE, Robbins KE, Brown TM et al. Failure of routine HIV-1 tests in a case involving transmission with preseroconversion blood components during the infectious window period. *JAMA* 2000; 284: 238–240.
16 Osborn JE, Shahidi NT. Thrombocytopenia in murine cytomegalovirus infection. *J Lab Clin Med* 1973; 81: 53.
17 Maciejewski JP, Bruening EE, Donahue RE, Mocarski ES, Young NS, Jeor SCS. Infection of hematopoietic progenitor cells by human cytomegalovirus. *Blood* 1992; 80: 170–178.
18 Mackow ER, Gavrilovskaya N. Cellular receptors and hantavirus pathogenesis. *Curr Top Microbiol Immunol* 2001; 256: 91–115.
19 Walz PH, Steficek BA, Baker JC, Kaiser L, Bell TG. Effect of experimentally induced type II bovine diarrhea virus infection on platelet function in calves. *Am J Vet Res* 1999; 60: 1396–1401.
20 Li X, Jeffers LJ, Garon C et al. Persistence of hepatitis C virus in a human megakaryoblastic leukemia cell line. *J Virol Hepat* 1999; 6: 107–114.
21 Li XM, Jeffers LJ, Shao LJ et al. Platelets are carriers of HCV RNA in patients with HCV infection. *Gastroenterology* 1994; 106: A930.
22 Silva M, Li XM, Cheinquer H et al. HCV-associated idiopathic thrombocytopenia purpura (ITP). *Gastroenterology* 1992; 102: A889.
23 Timuragaoglu A, Surucu F, Nalcaci M, Dincol G, Pekcelen Y. Anemia and thrombocytopenia due to parvovirus B-19 infection in a pregnant women. *J Med* 1997; 28: 245–249.
24 Schuttler GC, Caspari C, Jursch CA, WR WRW, Gerlich WH,

Cambridge Books Online © Cambridge University Press, 2009

Schaefer S. Hepatitis C virus transmission by a blood donation negative in nucleic acid amplification tests for viral RNA. *Lancet* 2000; 355: 41–42.

25 AuBuchon JP, Birkmeyer JD, Busch MP. Safety of the blood supply in the United States: opportunities and controversies. *Ann Intern Med* 1997; 127: 905–1009.

26 Schreiber GB, Busch MP, Kleinman SH, Korelitz JJ. The risk of transfusion-transmitted viral infections. *N Engl J Med* 1996; 334: 1685–1690.

27 Chan K. *Blood Supply: FDA Oversight and Remaining Issues of Safety.* US General Accounting Office, 1997.

28 Goldman M, Blajchman MA. Blood product-associated bacterial sepsis. *Transfu Med Revs* 1991; V: 73–83.

29 Morrow JF, Braine HG, Kickler TS, Ness PM, Dick JD, Fuller AK. Septic reactions to platelet transfusions: a persistent problem. *JAMA* 1991; 266: 555–558.

30 Blajchman MA, Ali A, Lyn P, Bardossy L, Richardson H. Bacterial surveillance of platelet concentrates: quantitation of bacterial load. *Transfusion* 1997; 37(Suppl): 74s.

31 Chiu EKW, Yuen KY, Lie AKW et al. A prospective study of symptomatic bacteremia following platelet transfusion and of its management. *Transfusion* 1994; 34: 950–954.

32 Wallace EL, Churchill WH, Surgenor DM. Collection and transfusion of blood components in the United States, 1992. *Transfusion* 1992; 35: 802–812.

33 Horowitz B, Wiebe ME, Lippin A, Stryker MH. Inactivation of viruses in labile blood derivatives I. Disruption of lipid-enveloped viruses by tri(n-butyl)phosphate detergent combinations. *Transfusion* 1985; 25: 516–522.

34 Heddle NM, Kalma L, Singer J et al. The role of plasma from platelet concentrates in transfusion reactions. *New Engl J Med* 1994; 331: 625–628.

35 Ohto H, Anderson KC. Survey of transfusion-associated graft-versus-host disease in immunocompetent recipients. *Transfus Med Revs* 1996; 10: 31–43.

36 Corash L. Photochemical decontamination of cellular blood components. *Anaesth Pharmacol Rev* 1995; 3: 138–149.

37 Edelson R, Berger C, Gasparro F et al. Treatment of cutaneous T-cell lymphoma by extracorporeal photochemotherapy. *N Engl J Med* 1987; 316: 297–303.

38 McEvoy MT, Stern RS. Psoralens and related compounds in the treatment of psoriasis. *Pharmacol Ther* 1987; 34: 75–97.

39 Alter HJ, Morel PA, Dorman BP et al. Photochemical decontamination of blood components containing hepatitis B and non-A, non-B virus. *Lancet* 1988; ii: 1446–1450.

40 Lin L, Wiesehahn GP, Morel PA, Corash L. Use of 8-methoxypsoralen and long wavelength ultraviolet radiation for decontamination of platelet concentrates. *Blood* 1989; 74: 517–525.

41 Dodd RY, Moroff G, Wagner S et al. Inactivation of viruses in platelet suspensions that retain their in vitro characteristics: comparison of psoralen-ultraviolet A and merocyanine 540-visible light methods. *Transfusion* 1991; 31: 483–490.

42 Margolis-Nunno H, Bardossy L, Robinson R, Ben-Hur E, Horowitz B, Blajchman MA. Psoralen-mediated photochemical decontamination of platelet concentrates: inactivation of cell-free and cell-associated forms of human immunodeficiency virus and assessment of platelet function in vivo. *Transfusion* 1997; 37: 889–895.

43 Goodrich RP, Yerram NR, Tay GB et al. Selective inactivation of viruses in the presence of human platelets: UV sensitization with psoralen derivatives. *Proc Natl Acad Sci USA* 1994; 91(12): 5552–5556.

44 Goodrich RP, Yerram NR, Crandall SL, Sowemimo-Coker SO. In vivo survival of platelets subjected to virus inactivation protocols using psoralen and coumarin photosensitizers. *Blood* 1995; 86 (Suppl 1): 354a.

45 Lin L, Cook DN, Wiesehahn GP et al. Photochemical inactivation of viruses and bacteria in platelet concentrates by use of a novel psoralen and long-wavelength ultraviolet light. *Transfusion* 1997; 37: 423–435.

46 Grass JA, Hei DJ, Metchette K et al. Inactivation of leukocytes in platelet concentrates by psoralen plus UVA. *Blood* 1998; 91: 2180–2188.

47 Knutson F, Alfonso R, Dupuis K et al. Photochemical inactivation of bacteria and HIV in buffy-coat-derived platelet concentrates under conditions that preserve in vitro platelet function. *Vox Sang* 2000; 78: 209–216.

48 Lin L, Corten L, Murthy KK, Corash L, Alter HJ. Photochemical inactivation of hepatitis B (HBV) and hepatitis C (HCV) virus in human platelet concentrates as assessed by a chimpanzee infectivity model. *Blood* 1998; 92(Suppl 1): 502a.

49 Eble B, Villena F, Chen C, Corash L. Photochemical inactivation of duck hepatitis B virus (DHBV) in human platelet concentrates: correlation of in vitro and in vivo effects. *Blood* 1993; 82 (Suppl 1): 205a.

50. Eble BE, Corash L. Photochemical inactivation of duck hepatitis B virus in human platelet concentrates: a model of surrogate human hepatitis B virus infectivity. *Transfusion* 1996; 36: 406–418.

51 Corash L, Behrman B, Rheinschmidt M et al. Post-transfusion viability and tolerability of photochemically treated platelet concentrates (PC). *Blood* 1997; 90(Suppl 1): 267a.

52 Slichter SJ, Corash L, Grabowski M et al. Viability and hemostatic function of photochemically treated (PCT) platelets in thrombocytopenic patients. *Blood* 1999; 94(Suppl 1): 376a.

53 vanRhenen D, Gulliksson H, Pamphilon D et al. S-59 Helinx photochemically treated platelets(plt) are safe and effective for support of thrombocytopenia: results of the EUROSPRITE Phase 3 trial. *Blood* 2000; 96(11): 819a.

54 Miller AB, Hoogstraten B, Staquet M, Winkler A. Reporting results of cancer treatment. *Cancer* 1981; 47: 207–214.

55 Londe H, Damonte P, Corash L, Lin L. Inactivation of human cytomegalovirus with psoralen and UVA in human platelet concentrates. *Blood* 1995; 86 (Suppl 1): 544a.

56 Lin L, Londe H, Hanson CV et al. Photochemical inactivation of cell-associated human immunodeficiency virus in platelet concentrates. *Blood* 1993; 82: 292–297.

57 Lin L, Londe H, Janda M, Hanson CV, Corash L. Photochemical inactivation of pathogenic bacteria in human platelet concentrates. *Blood* 1994; 83: 2698–2706.

Cambridge Books Online © Cambridge University Press, 2009

58 Benade LE, Shumaker J, Xu Y, Dodd R. Inactivation of free and cell-associated HIV in platelet suspensions by aminomethyltrimethyl (AMT) psoralen and ultraviolet light. *Transfusion* 1992; 32S: 33S.

59 Margolis-Nunno M, Williams B, Rywkin S, Horowitz B. Photochemical virus sterilization in platelet concentrates with psoralen derivatives. *Thromb Haemost* 1991; 65: 1162(abstract).

60 Rai S, Kasturi C, Grayzar J et al. Dramatic improvements in viral inactivation with brominated psoralens, naphthalenes and anthracenes. *Photochem Photobiol* 1993; 58(1): 59–65.

61 Yerram N, Forster P, Goodrich T et al. Comparison of virucidal properties of brominated psoralen with 8-methoxy psoralen (8–MOP) and aminomethyl trimethyl psoralen (AMT) in platelet concentrates. *Blood* 1993; 82 (Suppl 1): 402a.

62 Prodouz KN, Fratantoni JC, Boone EJ, Bonner RF. Use of laser-UV for inactivation of virus in blood products. *Blood* 1987; 70: 589–592.

63 Prodouz KN, Lytle CD, Keville EA, Budacz AP, Vargo S, Fratantoni JC. Inhibition by albumin of merocyanine 540-mediated photosensitization of platelets and viruses. *Transfusion* 1991; 31: 415–422.

64 Klein-Struckmeier A, Mohr H. Virus inactivation by methylene blue/light in thrombocyte concentrates. *Vox Sang* 1994; 67 (Suppl 2): 36.

65 Horowitz B, Rywkin S, Margolis-Nunno H et al. Inactivation of viruses in red cell and platelet concentrates with aluminum phthalocyanine (AlPc) sulfonates. *Blood Cells* 1992; 18: 141–50.

66 Goodrich RP. The use of riboflavin for the inactivation of pathogens in blood products. *Vox Sang* 2000; 78 Suppl 2: 211–215.

Cambridge Books Online © Cambridge University Press, 2009

53

Platelets and parasites

Michel Joseph

INSERM IFR 17, Institut Pasteur de Lille, France

Probably due to their long common evolution with their hosts, parasites have developed an efficient strategy to escape the immune mechanisms their presence induces. Especially with helminths, these mechanisms have the advantage of limiting the infection at a level which favours the host, and therefore the parasite, survival. Three decades of research in the field of antischistosome immunity has revealed that a large panel of immune functions, involving nearly all immune cell types, several cytokines or cell mediators, and the participation of specific antibodies of various isotypes[1] are required for the rapid and effective killing of infective larvae. In this context, antibody-dependent cell cytotoxicity constitutes the mainstay of killing mechanisms against helminths, with a particular involvement of the IgE isotype. The cellular part of ADCC covers a large panel of inflammatory cells, from mononuclear phagocytes (macrophages[2] and monocytes[3]) to neutrophils[4], eosinophils[5], or blood platelets[6]. It is difficult to assign to each participant its actual importance in the defence strategy of the immune system. The in vitro and ex vivo experiments have given a biased perspective of the involvement of each cell population, artificially amplifying their individual role. However, these investigations have allowed the demonstration of new functions for several inflammatory cell types, especially in the field of IgE responses, with a particular emphasis in allergic disorders.

This is particularly true for blood platelets, once thought to be exclusively involved in hemostasis and thrombosis, but now recognized as cells demonstrating a wide reactivity to soluble mediators and a complex metabolic behaviour beyond involvement in blood clotting or thrombus formation. Furthermore, the expression of IgE receptors on platelets gives a firm background to new concepts in the field of cell activation sustaining parasite immunity as well as inflammatory mechanisms. One striking example of the improvement in our knowledge of platelet behaviour is probably the nature of the IgE receptor involved in their killing functions. For 15 years, we assigned to FcεRII/CD23, the low affinity receptor for IgE, the effector structure supporting their cytotoxic activity against schistosomes[6]. More recent experiments, linked to the availability of new immunological reagents, have evidenced that the structure accounting for that property was FcεRI, the high affinity receptor for IgE[7]. The interesting aspect of such a mistake is the crucial and fascinating information it gives to the interactions between both receptor types. This will be discussed further in the present chapter. The IgE-dependent stimulation of platelets is, however, far from being the exclusive way for platelets to fight against parasites. There are many reports in the literature displaying the participation of platelets in physiopathological manifestations of parasitic diseases. The aim of this chapter is to discuss the involvement of platelets in the immune mechanisms which deal with parasites.

Thrombocytopenia

Among the features suggesting an involvement of platelets in parasitic diseases, the fall in platelet count is one of the best documented, particularly in protozoan infections. Thrombocytopenia is classically reported in malaria[8]. After anti-malarial treatment in a case of human infection by *Plasmodium vivax*, the level of platelets returned to normal[9], and similar observations were made in mice infected with *Plasmodium berghei*, where chloroquine sulfate cured the malaria and restored the platelet count to preinfection levels after a severe thrombocytopenia during acute infection[10]. The role of platelets in infections by micro-organisms was already recognized in the 1970s when partially digested *P. vivax* material was seen in platelets[11]. Investigations in mice have also shed light on a

possible beneficial role of blood platelets in malaria as experimentally induced thrombocytopenia in chronic and acute *Plasmodium berghei* murine malaria led to a greater mortality of thrombocytopenic mice compared to the control group[12]. However, thrombocytopenia may also be the consequence of a platelet sequestration, which could contribute to pathogenesis, such as in murine malaria[13,14].

Platelet stimulation against parasites

Several triggering conditions are able to induce a platelet stimulation leading to the death of parasites. Besides IgE, other compounds have been shown to participate to platelet immune functions including PDGF, C-reactive protein, substance P, IFNγ, TNF-α and β, thrombin and platelet-activating factor, IL-6 and C3b. Some antiparasitic drugs, such as diethylcarbamazine, may use platelets to kill their targets. In some situations, human platelets induce an in vitro inhibition of the growth of *Plasmodium falciparum* into erythrocytes without the apparent participation of specific antibody or components other than the parasite itself[15].

IgE

The primary demonstration of a platelet involvement in immunity to parasites was the IgE-mediated cytotoxicity[6]. Rats infected with *S. mansoni* presented an increasing thrombocytemia during the first 6 weeks of infestation. The concentration of platelets was raised up to three times their number in uninfected animals, and simultaneously the IgE concentration was increased. In parallel with cytotoxic properties appearing progressively in platelets isolated from infected rats, with a maximum at the sixth week of infection, sera from these animals were able to induce cytotoxicity in platelets from normal rats. Similarly, in the human, platelets isolated from patients with schistosomiasis were able to kill young larvae of schistosomes in vitro, and their serum able to stimulate the same properties in platelets from healthy subjects. IgE was the active isotype in this process as shown by the adsorption of the various subtypes of immunoglobulins, leading to the inhibition of killing only after IgE depletion, or by the induction of cytotoxicity on normal platelets by IgE monoclonal antibody against *S. mansoni*[16]. Preincubation of normal human platelets with a large excess of unrelated myeloma IgE, but not with IgG, prevented the induction of effector functions by sera from infected individuals[17]. A close contact between platelets and parasites was not necessary since the killing could be obtained through a 0.2 μm porosity filter separating the effector cells from the targets. This implicated the generation of soluble cytocidal mediators induced by the cross-linking of IgE by its specific ligand (parasite antigens for IgE from patients with schistosomiasis, allergens for IgE from allergic patients, or even anti-IgE antibody with IgE of various origins including myeloma IgE)[18]. Among the cytocidal mediators, cationic proteins could be excluded, at least as essential factors, since platelets from patients with grey-platelet syndrome, which lack granules containing such proteins, killed schistosomula as effectively as did normal platelets[19]. Catalase and superoxide dismutase were good inhibitors of the platelet cytocidal activity against schistosomes, emphasizing the hypothesis of a potential generation of oxygen-dependent free radicals[20]. IgE-stimulated platelets generated chemiluminescence in vitro, a process also suppressed by free radical scavengers. Aspirin and non-steroid anti-inflammatory drugs, inhibitors of cyclo-oxygenase, inhibited the IgE-mediated stimulation (cytotoxicity and chemiluminescence) of platelets. Platelets may also have a role in the extravasation and excretion of parasite eggs in schistosomiasis[21,22]. Suppression of platelet activity in *S. mansoni*-infected mice by a treatment with rabbit antimouse platelet serum or antiplatelet drugs induced a significant reduction of the parasite egg excretion.

Similar observations were made in filarial infections where normal rat platelets could be stimulated to kill *Dipetalonema viteae* microfilariae with IgE antibody in the serum of *D. viteae*-infected rats[23]. This antifilarial killing mechanism was neither stage nor species specific among filariae, in that microfilariae (L1) of *Brugia malayi* or of *Loa loa*, and infective larvae (L3) of *D. viteae* or of *B. malayi* were all killed by platelets incubated with anti-*D. viteae* IgE. Cross-reactivity among filarial antigens was the support of this widespread effect of *D. viteae* IgE. Platelets isolated from patients infected with *B. malayi* were also able to kill *B. malayi* microfilariae in vitro. As in the case of schistosome infections, platelets from normal donors acquired cytotoxic properties against the same targets in the presence of sera from filaria-infected patients, and the specific adsorption of IgE led to the abolition of cytotoxicity, strongly supporting the role of this isotype in the stimulating process[24].

The induction of an IgE-dependent protective response in rat toxoplasmosis has also been reported[25]. During *Toxoplasma gondii* infection, developed in the nude rat model, a significant increase of serum IgE was demonstrated, correlating with an increase of IgE-positive platelets. The participation of surface IgE in this effect was emphasized by the fact that only platelets bearing surface IgE were able to express a significant cytotoxicity towards

Cambridge Books Online © Cambridge University Press, 2009

tachyzoites in vitro, and a significant increase of the survival period when the cells were transferred to immunocompromized rats. It has also been reported that platelets can exert as strong a cytotoxic activity on *T. gondii* as cytotoxic T-lymphocytes, the release of thromboxane A2 being a crucial factor in this cytotoxic process[26].

PDGF

IgE, however, is not the only potential inducer of platelet cytotoxicity. It was observed that tachyzoites of *Toxoplasma gondii* induced the activation of human platelets and that platelets mediated the inhibition of intracellular growth in a virulent strain of *T. gondii*[27]. The role of platelet-derived growth factor was demonstrated by the use of human recombinant PDGF and of antihuman PDGF antibody which partially reversed its effect. The PDGF action was only evidenced on parasite-infected fibroblasts, not on free tachyzoites.

C-reactive protein

CRP, a major acute-phase component of inflammatory reactions, was shown to stimulate the secretion of cytotoxic mediators from normal platelets[28]. Normal rat platelets, incubated in rat serum recovered 24 h after the subcutaneous injection of turpentine, a known inducer of serum CRP, were able to kill schistosome larvae, whereas the serum either alone or in association with eosinophils or macrophages, had no activity in vitro on schistosomula. Human platelets treated with homologous serum obtained during an acute phase of inflammation or with purified C-reactive protein were able to kill the immature forms of the worm in vitro, whereas CRP-depleted serum was no more effective. Platelets treated with C-reactive protein were, furthermore, capable of conferring significant protection against schistosomiasis in transfer experiments. In radiolabelling experiments, it was demonstrated that 18% of the platelets in normal rats were CRP positive. These observations are in accordance with the fact that, in rats infected with *S. mansoni*, the concentration of C-reactive protein in the serum increases after the lung stage of infection and is at its highest at the time of terminal worm rejection. It is tempting to conclude that one feature of the worm rejection after week 4 of a primary infection in this species, which is non-permissive for schistosomes, is CRP and that platelets play a part in this process. Platelets from infected rats were larvicidal as early as day 14 after infection at a time when no antibody against schistosomes could be detected in the serum.

Substance P

Sensory neuropeptides, such as substance P, appear as potent mediators of various immunological reactions, and inhibit or stimulate a wide range of functions of immune inflammatory cells[29]. Substance P (SP) and its carboxy-terminal fragment (AA 4–11) induce the cytotoxic activity of platelets towards schistosomula, by 90% and 40%, respectively, whereas the modified C-terminal SP, the acid-free SP, exhibits no effect on the platelets. The neuropeptide effect occurred at low doses (10^{-8} M), and was specific as shown by inhibition studies with a substance P antagonist, the D-SP. Binding data obtained after flow cytofluorometry with FITC-SP led to the conclusion that SP binds specifically to about 20% of the homogenous population of platelets. Moreover, IgE could modulate the SP-dependent functions of platelets since the preincubation with myeloma human IgE leads to a dramatic decrease of the SP-dependent cytotoxic activity of platelets towards the larvae. These findings identify a potent mechanism for nervous system regulation of host defence responses.

Lymphocyte mediators

If platelets appear as competent immune cells, their activity can be modulated by T lymphocyte factors. They can be activated by supernatants of antigen or concanavalin A-stimulated $CD4^+$ T lymphocytes. Two factors were identified in such supernatants: IFNγ and TNF α or β. For IFNγ, its identity was shown by the neutralization of the induction of platelet cytotoxicity by a monoclonal anti-IFNγ antibody and the direct induction of a cytotoxic effect on platelets by pure recombinant IFNγ in a dose-dependent manner[30]. The presence of high affinity receptors for IFNγ on the platelet membrane has been reported[31]. A high affinity receptor for IFNγ has also been characterized on the surface of the human megakaryocytic cell line Dami[32]. In vivo experiments showed that the passive transfer of platelets preincubated with IFNγ conferred to animals infected 24 h later a significant level of protection compared to the level obtained after the transfer of normal untreated platelets[33]. The preincubation of immune platelets recovered from infected rats with IFNγ increased the level of protection normally obtained with immune cells alone. Similarly, an additive effect on platelet cytotoxicity was observed in vitro when IFNγ and IgE were tested together on the cells. As shown by cytofluorometric analysis, this effect was due in part to the increase of the expression of IgE receptors on platelets[34].

A second stimulating factor for platelets was identified in T-lymphocyte supernatants, after isoelectric focusing. This

Cambridge Books Online © Cambridge University Press, 2009

activity was neutralized by anti-TNF monoclonal antibody[35]. Both recombinant TNF-α and TNF-β mediated platelet cytotoxicity at very low concentrations and in a dose-dependent manner. An additive effect was observed in vitro between TNF-β and IFNγ. In vivo, TNF-α-stimulated platelets, transferred passively to normal rats, induced a significant level of protection against a challenge infection, not observed with platelets treated with IL-2, a lymphokine without stimulating properties for platelets[36].

The inhibiting functions of T-lymphocytes could be shown with a platelet activity suppressive lymphokine (PASL), produced by mitogen-stimulated $CD4^-/CD8^+$ lymphocytes, which inhibited the IgE-dependent platelet cytotoxicity[24]. Furthermore, antigen-specific T lymphocytes from *S. mansoni*- or *B. malayi*-infected patients generated a factor inhibiting the cytotoxicity of platelets from the same individuals against larvae of the corresponding parasites[37,38]. This suppressive lymphokine had a molecular weight of 15 000 to 20 000, a pI of 4.6, was heat and acid stable, sensitive to trypsin and proteinase K, but was insensitive to neuraminidase. It totally abolished the protection normally conferred against a challenge infection by the passive transfer of platelets from immune animals to normal rats by a preincubation of transferred platelets with PASL[39]. In an attempt to clone the PASL, a cDNA coding for ubiquitin was isolated. Like PASL, purified ubiquitin can inhibit the cytotoxic properties of platelets and the production of oxygen metabolites by these cells[40]. PASL and ubiquitin also share similar physicochemical characteristics (acid and heat sensitivity, stability) and show an absence of species specificity: human PASL was able to inhibit the effector functions of human, and also of rat and mouse platelets and vice versa. Purified bovine ubiquitin acts similarly on human, rat or mouse platelets. A binding structure for ubiquitin exists on blood platelets. The only discrepancy observed was that a rabbit polyclonal antibody directed against ubiquitin which neutralized the inhibiting activity of ubiquitin on platelets did not do so with lymphocyte supernatants. This could be due to determinants recognized on ubiquitin possibly absent or modified on PASL. Nonetheless, provisionally, one can accept that PASL probably belongs to the family of ubiquitins.

Interleukin 6

A co-operation between monocytes and platelets for the killing of *S. mansoni* was also described[41]. Supernatants obtained after a 24 h adherence of normal human monocytes were able to induce, in a concentration dependent manner, the cytotoxicity of normal human platelets against schistosomula in vitro. The physicochemical characteristics of the factor suggested a role for IL-6, detected in such supernatants, in the activity. The hypothesis was confirmed by the neutralization of the cytotoxic effect by a polyclonal serum against IL-6, whereas polyclonal sera against IL-1β or TNF-α, also present in the medium, were inactive. Recombinant human IL-6 induced the cytotoxic functions of platelets, demonstrating a direct effect of this cytokine on blood platelets.

C3b

Investigations in mice infected with *Trypanosoma musculi* have shown an in vitro interaction between platelets and parasites, leading to the death of trypanosomes within a few minutes and their lysis[42]. The role of IgG antibody and C3b has been suggested in this mechanism[43], as well as in the clearance of promastigotes from *Trypanosoma cruzi* from the blood stream by platelets[44,45]. The group of Gresele and colleagues has brought recent support to the participation of platelets in cytocidal effects against *Trypanosoma equiperdum*, showing that this horse parasite, which can be used in experimental infections of mice, is killed in vitro by platelets, and that IgM (not C3) enhances platelet trypanocidal activity[46]. However the possibility that, in these infections, platelets could participate in pathology rather than induce protection must be kept in mind. This is true for Chagas disease[47] as well as for African trypanosomiasis[48,49].

Thrombin and platelet activating factor

We observed that, in vitro, thrombin and platelet-activating factor (PAF), two classical agonists of platelet aggregation, mediated either cytotoxicity at low concentrations or aggregation at higher concentrations, with no overlapping of the respective ranges[50]. These two effects could never be produced simultaneously. Killing functions and aggregating properties seem therefore to be two distinct mechanisms. It was also shown that triggering platelets with low concentrations of one aggregating agent, or with a cytotoxicity inducer (IgE), impaired very significantly the aggregation mediated by the other agent, ruling out receptor tachyphylaxis. These two activities appeared as distinct and antithetical. However, their induction might be the consequence of triggering the same receptors with different intensity, since PAF-induced, but not thrombin-induced, cytotoxicity could be inhibited with specific PAF-antagonists BN 52021 and BN 52024 also known to inhibit PAF-induced aggregation. The concept of a dual antinomic function of platelets is strengthened by

Cambridge Books Online © Cambridge University Press, 2009

the possibility of inducing cytotoxic expression against schistosome larvae with purified platelet membranes stimulated with IgE/anti-IgE or IgE/allergen. Aggregation inhibited almost completely the IgE-dependent cytotoxic process, even with platelet membranes from disrupted aggregates, whereas membranes from disrupted unaggregated platelets could express significant cytotoxicity. Therefore, as these effects could neither be produced successively nor simultaneously, it implies a diversion in the metabolic processes leading to one or the other function. This is the reason why we have proposed the term of platelet 'stimulation' for the induction of cytotoxicity to avoid confusion with platelet 'activation' which commonly points to aggregation.

Diethylcarbamazine

The mode of action of a 50-year-old antifilarial drug, diethylcarbamazine (DEC), has remained obscure despite widespread use and intensive investigations. The marked contrast between an extremely rapid action in vivo and the absence of any significant activity on microfilariae in vitro is unique among chemotherapeutic agents. We have reported that the effect of DEC could be mediated by blood platelets, with the additional triggering of a filarial excretory antigen (FEA)[51]. Platelets isolated from individuals treated orally with DEC were cytotoxic in vitro for microfilariae, and so were platelets from healthy donors treated in vitro with the drug. Fifteen days after oral administration, platelets were still active against microfilariae, whereas after 48 h free DEC was undetectable in sera, suggesting that the target of DEC was the platelet population and, more likely, their bone marrow precursors, as the effect of DEC outlasted the average platelet lifetime of 9–10 days. However, the observation that such platelets were inactive against schistosomula, unless microfilariae were introduced into the assay, suggested a specific signal coming from the target towards DEC-treated platelets. Support for this hypothesis came from experiments in which a filarial excretory antigen triggered the cytotoxicity against schistosomula incubated with DEC-treated platelets. Among the mediators of the killing process, oxygen free radicals have been proposed, since scavengers of activated oxygen species (sodium formiate, mannitol, uric acid, sodium salicylate and sodium azide) inhibited the platelet killing activity. Other toxic intermediates were probably involved, such as lipoperoxides, as shown by the amplification of the killing by transition metals (Fe^{2+} down to 10^{-11} M) and its abolition by iron chelators (*o*-phenanthroline).

All these stimuli probably triggered platelets by different pathways but achieved the same result: the generation of molecules expressing cytocidal properties for parasites. A common feature of these various signals was the induction of cytotoxic properties without aggregation, suggesting the stimulation of a platelet compartment different from that activated in the classical process of aggregation. This justifies the distinct denomination of stimulation and activation for these two opposite processes. We would now like to present the most surprising observation that these investigations have brought about during the last few years, in the field of the IgE-mediated stimulation of blood platelets.

Two interactive receptors for IgE

FcεRI and CD23

It is obvious today that the investigations dedicated to immune mechanisms against the helminth parasite *S. mansoni* in mammals have led to the demonstration and to original functions of IgE receptors on inflammatory cells, giving them properties other than their exclusive participation to immediate hypersensitivity reactions linked to mast cells and basophils[52]. Over two decades, we have contributed research describing, on rat peritoneal macrophages, human monocytes and alveolar macrophages, eosinophils and blood platelets in rat and man, a low affinity receptor for IgE, FcεRII, later on recognized as CD23. The binding of parasite-specific antibodies to the surface of these cells mediated cytotoxic properties for larvae of the pathogen in vitro and also in vivo as shown by the protection conferred to naïve recipients by the transfer of cells loaded with parasite-specific IgE. Very similar observations, particularly with blood platelets, were reported during that period against other metazoan[23] and protozoan parasites[25].

It is important to underline here that, if effector functions of inflammatory cells mediated by IgE are expressed through the killing of schistosomula and the larvae of *S. mansoni*, the triggering process for the cell stimulation is not necessarily provided by antiparasitic IgE. Inflammatory cells from allergic patients, bearing allergen-specific IgE, generate antischistosome cytotoxic mediators if the specific allergen is introduced into the assay. This means that, if the activating signal is specific, i.e. IgE + specific allergen, its expression is not, i.e. death of parasites unrelated to sensitizing IgE. Furthermore, cells from normal individuals may be sensitized in vitro with IgE from allergic or parasitized patients and then behave as cells isolated from individuals with the corresponding pathology.

Cambridge Books Online © Cambridge University Press, 2009

During that period, the availability of polyclonal and monoclonal antibodies limited only to the FcεRII/CD23 receptor led us to assign to that receptor the structure responsible for the binding, and therefore for the effector function of IgE on inflammatory cells. Such a conclusion was drawn from the observation that anti-CD23 antibodies inhibited both the binding of IgE on the concerned cells and the cytotoxicity they expressed in vitro against parasites. That concept now appears to be partially wrong, since in the last decade it has been demonstrated that when specific antibodies to the human high affinity receptor for IgE, FcεRI, were made available, such antibodies were also able to influence platelet function. FcεRI, and mRNA for its three different chains, were found on the same inflammatory cells: mononuclear phagocytes[53,54], eosinophils[55], blood platelets[7,56], as well as on Langerhans and dendritic cells[57,58]. Furthermore, the results obtained with eosinophils and platelets conferred to the high affinity receptor for IgE the status of the actual effector structure, until then attributed to CD23. We had therefore to understand why we misinterpreted the inhibitory effects of anti-CD23 antibodies in the killing functions of platelets, if really FcεRI was doing the job.

Recently, blood platelets led us to bring the beginning of an explanation to this apparent contradiction on the respective role of CD23 and FcεRI in the IgE-mediated cytotoxicity. As indicated above, polyclonal and monoclonal antibodies to CD23 inhibited the binding of IgE to platelets and the cytotoxicity expressed in vitro. One detail was nevertheless surprising: if the cross-linking of adjacent receptors, loaded with IgE, by specific antigens was the starting signal for the cytotoxicity, why could none of the anti-CD23 used alone mimic the process, when their divalence should have induced the killing effect. In 1994 we could use a monoclonal antibody directed towards the binding domain of IgE on the alpha chain of the FcεRI (mAb 15–1, produced by Jean-Pierre Kinet, Boston). Besides the demonstration of the presence of this receptor on platelets, this antibody inhibited the binding of IgE to platelets, its Fab monovalent fragment inhibited their IgE-induced cytotoxicity, and, above all, the antibody, either in its IgG form or in its F(ab′)2 form, triggered the effector function of platelets on parasites. We had got finally an antibody able to mimic the stimulation process of an allergen-dependent cross-linking of adjacent IgE receptors, as observed for decades in the degranulation of mast cells and basophils. Last but not least, the anti-CD23 antibody inhibited the platelet cytotoxicity induced by divalent anti-FcεRI, as efficiently as it inhibited the cytotoxicity induced by IgE. The inhibition could be obtained if anti-CD23 was added to the platelet before or at the same time as anti-FcεRI or IgE, but not after. The conclusion, for blood platelets, is that the stimulation of effector functions by IgE, generating cytocidal mediators, is linked to the high affinity receptor for IgE, FcεRI, and that the low affinity receptor, CD23, exerts a regulatory role on the effector functions of the former. Investigations carried out these last few years have brought some light on the underlying mechanism.

The regulatory role of CD23 on FcεRI signals via inhibitory phosphatases

In its intracytoplasmic N-terminus, human CD23 presents an immunoreceptor tyrosine-based inhibitory motif (ITIM), which could be involved in the inhibitory effect observed with anti-CD23. On the basis of the known amino-acid sequence of human CD23, we synthesised a trideca-peptide containing the inhibitory motif (Tyr–Ser–Leu–Leu or Y–S–L–L). Its lysine-phosphorylated form (Y–S–L–pL), which is produced during the physiological activation of CD23, is able to capture, in a human platelet lysate, a phosphatase which plays a part in the inhibition of transduction signals of activated cells. This phosphatase, called SHIP (for SH2–Inositol-5–Phosphatase) blocks calcium influx in the activation process of cells. It seems that two other inhibiting phophatases, which dephosphorylate other links of the transduction cascade, SHP-1 and SHP-2, are not captured by the phosphorylated ITIM of CD23. If these preliminary observations are confirmed, they bring a new light on the role of SHIP, the presence of which in platelets has been reported recently, but without a defined function until now in platelet stimulation or aggregation.

A second element opens interesting perspectives to the understanding of CD23–FcεRI interactions. We observed that soluble CD23 could inhibit the cytotoxic properties of platelets mediated by IgE or by anti-FcεRI. The inhibition was as strong as that obtained with anti-CD23 antibody, with very low concentrations of soluble CD23 (25 pg/ml). Soluble CD23 was unable to inhibit a non-IgE dependent pathway of platelet stimulation, also described in our laboratory, induced by thrombin. The effect of soluble CD23 seems therefore limited to IgE functions of platelets. The problem is to define the binding structure on platelet surface on which soluble CD23 exerts its inhibitory effect. Human CD23 bears, in its C-terminal part a DGR peptide (reverse of RGD) susceptible to bind lectins. We had the opportunity of getting mutant soluble CD23, devoid of DGR sequence. This RGD-free soluble CD23 lost its inhibiting activity on the cytotoxic functions of platelets induced by IgE or anti-FcεRI. This sequence is therefore potentially involved in the binding of CD23 to a platelet

Cambridge Books Online © Cambridge University Press, 2009

structure leading to inhibitory action (CD11b? CD21? Other?). It is not demonstrated that both mechanisms, that induced by anti-CD23, and that induced by soluble CD23, are based on the same patterns. One hypothesis would be that anti-CD23, by preventing IgE binding to CD23 and therefore stabilizing CD23 against autocatalysis, would increase the release of soluble CD23 or even induce it, leading to the inhibition observed. If this mechanism has some correlate in other cells implicated in immediate hypersensitivity, especially mast cells and basophils, it might open interesting new approaches in the treatment or prevention of allergic disorders.

A general feature of the platelet metabolism in IgE-dependent reactions

We would like to conclude this overview by mentioning the extensions that the anti-parasite immunity supported by platelets have gained in other fields of human and experimental pathology. The most valuable opening of our observations was that directed towards allergic diseases, with the concept of a potential direct involvement of these blood elements in either stage of the physiopathologic mechanisms of IgE-mediated allergy (especially allergic asthma to inhaled allergens or systemic anaphylactic reactions to hymenoptera venom) and drug-induced intolerance (such as aspirin-induced asthma). Another in this volume illustrates this aspect. The whole of these investigations results in the definite integration of blood platelets into the complex network of cell interactions in various immunological and inflammatory reactions or disorders. Such a benefit alone is worth the decades of research on that topic.

References

1 Capron A, Dombrowicz D, Capron M. Regulation of the immune response in experimental and human schistosomiasis: the limits of an attractive paradigm. *Microbes Infect* 1999; 1: 485–490.

2 Capron A, Dessaint JP, Capron M, Bazin H. Specific IgE antibodies in immune adherence of normal macrophages to *Schistosoma mansoni* schistosomules. *Nature* 1975; 253: 474–475.

3 Joseph M, Capron A, Butterworth AE, Sturrock RF, Houba V. Cytotoxicity of human and baboon mononuclear phagocytes against schistosomula in vitro: induction by immune complexes containing IgE and *Schistosoma mansoni* antigens. *Clin Exp Immunol* 1978; 33: 48–56.

4 Dean DA, Wistar R, Chen P. Immune response of guinea pigs to *Schistosoma mansoni.* I. In vitro effects of antibody and neutrophils, eosinophils and macrophages on schistosomula. *Am J Trop Med Hyg* 1975; 24: 74–82.

5 Capron M, Rousseaux J, Mazingue C, Bazin H, Capron A. Rat mast cell–eosinophil interaction in antibody-dependent eosinophil cytotoxicity to *Schistosoma mansoni* schistosomula. *J Immunol* 1978; 121: 2518–2525.

6 Joseph M, Auriault C, Capron A, Vorng H, Viens P. A new function for platelets: IgE-dependent killing of schistosomes. *Nature* 1983; 303: 810–812.

7 Joseph M, Gounni AS, Kusnierz JP et al. Expression and functions of the high-affinity IgE receptor on human platelets and megakaryocyte precursors. *Eur J Immunol* 1997; 27: 2212–2218.

8 Horstmann RD, Dietrich M, Bienzle U, Rasche H. Malaria-induced thrombocytopenia. *Blut* 1981; 42: 157–164.

9 Yamaguchi S, Kubota T, Yamagishi T et al. Severe thrombocytopenia suggesting immunological mechanisms in two cases of vivax malaria. *Am J Hematol* 1997; 56: 183–186.

10 Inyang AL, Okpako DT, Essien EM. Platelet reactions in acute *Plasmodium berghei* infection in Swiss albino mice. *Haematologia* 1987; 20: 101–108.

11 Fajardo LF. The role of platelets in infections. I. Observations in human and murine malaria. *Arch Pathol Lab Med* 1979; 103: 131–134.

12 Polack B, Delolme F, Peyron F. Protective role of platelets in chronic (Balb/C) and acute (CBA/J) *Plasmodium berghei* murine malaria. *Haemostasis* 1997; 27: 278–285.

13 Grau GE, Lou JN. Experimental cerebral malaria: possible new mechanisms in the TNF-induced microvascular pathology. *Soz Praventivmed* 1995; 40: 50–57.

14 Piguet PF, Da Laperrousaz C, Vesin C, Tacchini-Cottier F, Senaldi G, Grau GE. Delayed mortality and attenuated thrombocytopenia associated with severe malaria in urokinase- and urokinase receptor-deficient mice. *Infect Immun* 2000; 68: 3822–3829.

15 Peyron F, Polack B, Lamotte D, Kolodie L, Ambroise-Thomas P. *Plasmodium falciparum* growth inhibition by human platelets in vitro. *Parasitology* 1989; 99 Pt 3: 317–322.

16 Verwaerde C, Joseph M, Capron M et al. Functional properties of a rat monoclonal IgE antibody specific for *Schistosoma mansoni. J Immunol* 1987; 138: 4441–4446.

17 Joseph M, Capron A, Ameisen JC et al. The receptor for IgE on blood platelets. *Eur J Immunol* 1986; 16: 306–312.

18 Joseph M, Auriault C, Capron M et al. IgE-dependent platelet cytotoxicity against helminths. *Adv Exp Med Biol* 1985; 184: 23–33.

19 Ameisen JC, Joseph M, Caen JP et al. A role for glycoprotein IIb–IIIa complex in the binding of IgE to human platelets and platelet IgE-dependent cytotoxic functions. *Br J Haematol* 1986; 64: 21–32.

20 Joseph M. The generation of free radicals by blood platelets. In Joseph M, ed. *Immunopharmacology of Platelets.* London: Academic Press; 1995: 209–225.

Cambridge Books Online © Cambridge University Press, 2009

21 Ngaiza JR, Doenhoff MJ. Blood platelets and schistosome egg excretion. *Proc Soc Exp Biol Med* 1990; 193: 73–79.
22 Ngaiza JR, Doenhoff MJ, Jaffe EA. *Schistosoma mansoni* egg attachment to cultured human umbilical vein endothelial cells: an in vitro model of an early step of parasite egg excretion. *J Infect Dis* 1993; 168: 1576–1580.
23 Haque A, Cuna W, Bonnel B, Capron A, Joseph M. Platelet mediated killing of larvae from different filarial species in the presence of *Dipetalonema viteae* stimulated IgE antibodies. *Parasite Immunol* 1985; 7: 517–526.
24 Pancré V, Auriault C, Joseph M, Cesbron JY, Kusnierz JP, Capron A. A suppressive lymphokine of platelet cytotoxic functions. *J Immunol* 1986; 137: 585–591.
25 Ridel PR, Auriault C, Darcy F et al. Protective role of IgE in immunocompromised rat toxoplasmosis. *J Immunol* 1988; 141: 978–983.
26 Yong EC, Chi EY, Fritsche TR, Henderson WR. Human platelet-mediated cytotoxicity against *Toxoplasma gondii*: role of thromboxane. *J Exp Med* 1991; 173: 65–78.
27 Chumpitazi BF, Simon J, Polack B et al. Human platelet inhibition of *Toxoplasma gondii* growth. *Clin Exp Immunol* 1998; 111: 325–333.
28 Bout D, Joseph M, Pontet M, Vorng H, Deslée D, Capron A. Rat resistance to schistosomiasis: platelet-mediated cytotoxicity induced by C-reactive protein. *Science* 1986; 231: 153–156.
29 Damonneville M, Monté D, Auriault C, Capron A. The neuropeptide substance P stimulates the effector functions of platelets. *Clin Exp Immunol* 1990; 81: 346–351.
30 Pancré V, Joseph M, Mazingue C, Wietzerbin J, Capron A, Auriault C. Induction of platelet cytotoxic functions by lymphokines: role of interferon-gamma. *J Immunol* 1987; 138: 4490–4495.
31 Molinas E, Wietzerbin J, Falcoff M. Human platelets possess receptors for a lymphokine: demonstration of high specific receptors for huIFNgamma. *J Immunol* 1987; 138: 802–806.
32 Monté D, Wietzerbin J, Pancré V et al. Identification and characterization of a functional receptor for interferon-gamma on a megakaryocytic cell line. *Blood* 1991; 78: 2062–2069.
33 Pancré V, Schellekens H, Van der Meide P et al. Biological effect of interferon-gamma during the course of experimental infection of rat by *Schistosoma mansoni*. *Cell Immunol* 1990; 125: 58–64.
34 Pancré V, Joseph M, Capron A et al. Recombinant human interferon-gamma induces increased IgE receptor expression on human platelets. *Eur J Immunol* 1988; 18: 829–832.
35 Damonneville M, Wietzerbin J, Pancré V et al. Recombinant tumor necrosis factors mediate platelet cytotoxicity to *Schistosoma mansoni* larvae. *J Immunol* 1988; 140: 3962–3965.
36 Damonneville M, Pancré V, Capron A, Auriault C. Protection of rats against *Schistosoma mansoni* infection induced by platelets stimulated with the murine recombinant tumor necrosis factor alpha. *Int Arch Allergy Appl Immunol* 1990; 92: 361–363.
37 Pancré V, Cesbron JY, Joseph M, Barbier M, Capron A, Auriault C. IgE-dependent killing of *Schistosoma mansoni* schistosomula by human platelets: modulation by T cell products. *Int Arch Allergy Appl Immunol* 1988; 87: 371–375.
38 Pancré V, Cesbron JY, Auriault C, Joseph M, Chandenier J, Capron A. IgE-dependent killing of *Brugia malayi* microfilariae by human platelets and its modulation by T cell products. *Int Arch Allergy Appl Immunol* 1988; 85: 483–486.
39 Pancré V, Joseph M, Capron A, Delanoye A, Vorng H, Auriault C. Characterization of a suppressive factor of platelet cytotoxic functions in human and rat *Schistosomiasis mansoni*. *Clin Exp Immunol* 1989; 76: 417–421.
40 Pancré V, Pierce RJ, Fournier F et al. Effect of ubiquitin on platelet functions: possible identity with platelet activity suppressive lymphokine (PASL). *Eur J Immunol* 1991; 21: 2735–2741.
41 Pancré V, Monté D, Delanoye A, Capron A, Auriault C. Interleukin-6 is the main mediator of the interaction between monocytes and platelets in the killing of *Schistosoma mansoni*. *Eur Cytokine Netw* 1990; 1: 15–19.
42 Viens P, Dubois R, Kongshavn PA. Platelet activity in immune lysis of *Trypanosoma musculi*. *Int J Parasitol* 1983; 13: 527–530.
43 Shaw KT, Mawji Y, Stevenson MM, Kongshavn PA. Cells within the vascular system capable of mediating trypanocidal activity in vitro. *Infect Immun* 1991; 59: 3143–3150.
44 Takehara HA, Mota I. The possible mechanism of action of IgG antibodies and platelets protecting against *Trypanosoma cruzi* infection. *Braz J Med Biol Res* 1991; 24: 759–765.
45 Umekita LF, Piazza RM, Mota I. Role of platelets and complement in the clearance of epimastigote forms of *Trypanosoma cruzi*. *Braz J Med Biol Res* 1994; 27: 2391–2399.
46 Momi S, Perito S, Mezzasoma AM, Bistoni F, Gresele P. Involvement of platelets in experimental mouse trypanosomiasis: evidence of mouse platelet cytotoxicity against *Trypanosoma equiperdum*. *Exp Parasitol* 2000; 95: 136–143.
47 Tanowitz HB, Burns ER, Sinha AK et al. Enhanced platelet adherence and aggregation in Chagas' disease: a potential pathogenic mechanism for cardiomyopathy. *Am J Trop Med Hyg* 1990; 43: 274–281.
48 Nwagwu M, Inyang AL, Molokwu RI, Essien EM. Platelet-aggregating activity of released factor(s) from *Trypanosoma brucei brucei*. *Afr J Med Med Sci* 1989; 18: 283–287.
49 Davis CE. Thrombocytopenia: a uniform complication of African trypanosomiasis. *Acta Trop* 1982; 39: 123–133.
50 Tran A, Vanhée D, Capron A, Vorng H, Braquet P, Joseph M. Separate induction of human blood platelet aggregation or cytotoxicity by different concentrations of PAF-acether and thrombin. *Agents Actions* 1992; 36: 39–43.
51 Cesbron JY, Capron A, Vargaftig BB et al. Platelets mediate the action of diethylcarbamazine on microfilariae. *Nature* 1987; 325: 533–536.
52 Capron M, Capron A. Immunoglobulin E and effector cells in schistosomiasis. *Science* 1994; 264: 1876–1877.
53 Maurer D, Fiebiger E, Reininger B et al. Expression of functional high affinity immunoglobulin E receptors (Fc epsilon RI) on monocytes of atopic individuals. *J Exp Med* 1994; 179: 745–750.
54 Ochiai K, Kagami M, Umemiya K, Matsumura R, Kawashima T, Tomioka H. Expression of high-affinity IgE receptor (Fc epsilon RI) on human alveolar macrophages from atopic and

Cambridge Books Online © Cambridge University Press, 2009

non-atopic patients. *Int Arch Allergy Immunol* 1996; 111: 55–58.
55 Gounni AS, Lamkhioued B, Ochiai K et al. High-affinity IgE receptor on eosinophils is involved in defence against parasites. *Nature* 1994; 367: 183–186.
56 Hasegawa S, Pawankar R, Suzuki K et al. Functional expression of the high affinity receptor for IgE (FcepsilonRI) in human platelets and its intracellular expression in human megakaryocytes. *Blood* 1999; 93: 2543–2551.
57 Bieber T. Fc epsilon RI on human Langerhans cells: a receptor in search of new functions. *Immunol Today* 1994; 15: 52–53.
58 Maurer D, Fiebiger S, Ebner C et al. Peripheral blood dendritic cells express Fc epsilon RI as a complex composed of Fc epsilon RI alpha- and Fc epsilon RI gamma-chains and can use this receptor for IgE-mediated allergen presentation. *J Immunol* 1996; 157: 607–616.

Cambridge Books Online © Cambridge University Press, 2009

54

Platelets and tumours

Maria Benedetta Donati and Virgilio Evangelista

Department of Vascular Medicine and Pharmacology, Istituto di Ricerche Farmacologiche Mario Negri, Santa Maria Imbaro, Italy

Introduction

Despite pioneer reports in the nineteenth century[1,2] the interactions between the components of the hemostatic system and cancer have so far been studied without a really integrated approach and no true experimental–clinical cross-talk has been established.

Tumour growth and dissemination is a multistep process which goes from oncogenic transformation to progression and local growth, vascularization, detachment of tumour cells from the primary and invasion of capillary or lymphatic vessels, transport, arrest in capillary beds, adhesion to vessel wall, extravasation and growth at distant sites[3]. All these steps involve different interactions with the components of the hemostatic system, which have been clarified mainly in in vitro approaches during the past 30 years[4–7]. Tumour cells have been demonstrated as the source of procoagulant, fibrinolytic, platelet aggregation activities, adhesion processes with leukocytes and endothelial cells, and interactions with tumour-associated macrophages. In particular, clinical and experimental evidence suggests that blood platelets are involved at several steps of the tumour dissemination cascade[8,9]. In this sequence of events, the adhesion of tumour cells to the vessel wall seems to be favoured by the gathering of activated *platelets* in the surroundings. More than 40 years ago, platelet–tumour cell emboli were observed in microcinematography experiments in rabbits and human pathological specimens, suggesting the possible involvement of platelets in tumour dissemination[10]. The discovery that thrombocytopenia, induced by antiplatelet serum or neuroaminidase treatment, reduced experimental metastasis in mice, further supported this hypothesis[11,12]. Since then, various antiplatelet drugs have been used for metastasis treatment and the interactions between cancer cells and platelets have been repeatedly investigated[8,9,12,13]. However, neither the determinants of platelet involvement in metastasis nor the mechanisms underlying these interactions have been completely clarified.

It is the purpose of this chapter to revisit some of the platelet–tumour cell interactions established in the early studies with the perspective of more recent acquisitions, mainly in terms of platelet/leukocyte leukocyte/endothelial cell receptors and cross-talk, a field which has rapidly grown since the 1980s.

While 40–50 years ago platelet–tumour cell interactions were probably the first example of cell–cell interactions described in some detail, we are now in a position to follow the process of tumour cell arrest and extravasation with models taken from the sequence of events involving leukocyte recruitment, arrest and tissue penetration at an inflammatory site[14].

The study of platelet/tumour cell interactions may have a dual implication: on one hand, platelet behaviour may be modified by tumour growth, as part of the host response to the tumour, on the other, platelets may play themselves a pathogenetic role in tumour growth and dissemination.

Platelet changes associated with malignancy

The involvement of blood platelets during growth and dissemination of tumours may be mainly inferred from experimental studies; such studies have used different models and the results obtained may vary greatly, depending on the type of tumour and the route of administration of tumour cells[4,15]. Among the models of metastasis that have been developed, two have been more extensively studied in experimental animals.

(i) The intravenous (i.v.) injection of tumour cells in mice, which leads, within some weeks, to development of tumour nodules in the lung. This model is also referred

to as 'artificial' metastasis, since huge amounts of tumour cells are given to a previously healthy animal in the absence of all those metabolic and biochemical changes generally associated with the growth of a solid tumour in clinical conditions. On the other hand, this model more closely mimics metastasis formation which may occur following sudden spreading of tumour cells in the circulation of patients undergoing surgical manipulations for tumour removal.

(ii) The subcutaneous (s.c.) or intramuscular (i.m.) injection of tumour cells, which leads to the growth of a primary tumour at the site of implantation and to the subsequent dissemination to distant organs, where metastatic nodules may grow. This model is termed 'spontaneous' metastasis, since its development mimics more closely, than that of the lung colony model, the clinical evolution in patients with solid tumours from which tumour cells gradually migrate to distant organs.

The involvement of the host's hemostatic system, as well as the response to pharmacological modulation (see later), may be completely different in these two models.

We shall give some examples for this concept, taken from the experience our group has gathered mainly in murine tumours, such as the Lewis Lung Carcinoma, widely used for preclinical chemotherapy studies both in Europe and in the USA.

When tumour cells are injected i.v. in mice ('artificial model'), a marked thrombocytopenia occurs as a part of an intravascular coagulation syndrome, characterized also by low fibrinogen levels and by high levels of fibrinogen degradation products. Thrombocytopenia can be detected 2–5 minutes after tumour cell injection and is rapidly reversible. The fall in platelet count is presumably due to platelet aggregation in response to thrombin, developing from the procoagulant activities of tumour cells. Tumour cells can induce in vivo thrombocytopenia independently from their direct platelet aggregatory properties; moreover, in vivo platelet consumption can be prevented not only by antiplatelet agents, but also, and even better, by the treatment of animals with the anticoagulant warfarin[16–18]. Tumour cell-induced thrombocytopenia does not occur in mice with a congenital deficiency of platelet function due to defective content of their dense granules, the so-called 'beige' mice[8].

The above-mentioned changes are very short-lived and are reversible within 1 hour of tumour cell injection. The subsequent development of lung colonies is not accompanied by any hematological changes; normal platelet and red blood cell counts and fibrinogen levels are measured during the growth of 'artificial' metastases[15].

In contrast, several changes occurring during 'spontaneous' metastases have been described in the Lewis Lung Carcinoma, the JW sarcoma and the mFS6 fibrosarcoma in mice[16,19,20]. The platelet changes occurring during development of Lewis Lung Carcinoma implanted i.m. have been extensively studied[16]. Mild intravascular coagulation, with microangiopathic hemolytic anaemia, thrombocytopenia and raised fibrinogen levels with increased fibrinogen turnover were observed during tumour growth. Thrombocytopenia was not accompanied by increase in platelet turnover nor by changes in the splenic pool; bone marrow examination and indirect pharmacological data further confirmed that it was rather due to impaired platelet production than to enhanced intravascular consumption. Thrombocytopenia was not abolished by chronic treatment with antiplatelet or anticoagulant agents; the lack of a clear pathogenetic link with metastasis formation was suggested also by the observation that tumour-bearing animals treated with warfarin showed a marked inhibition of metastases[15,16]. In conclusion, thrombocytopenia appeared to be related to the presence of the primary tumour as also indicated by normal platelet counts in mice whose primary tumour was surgically removed when metastatic growth had already started[15]. It is thus conceivable that thrombocytopenia under these conditions could be favoured by the release of some, as yet undefined, toxic factors from the primary tumour, which would exert a myelotoxic effect.

Since the 1980s new experimental systems have been used to study the biological processes related to cancer metastasis: in particular, 'metastatic variants', i.e. sublines obtained through in vitro/in vivo passages from the same parental line, characterized by marked differences in their metastatic potential in either spontaneous or artificial models of dissemination, have been developed[3]. We studied the platelet involvement in mFS6 fibrosarcoma, a benzopyrene-induced tumour, and in several metastatic sublines derived from its lung metastasis nodules; the potential for spontaneous metastasis of the sublines ranged from 0 to 100% incidence of lung nodules. Gradual thrombocytopenia developed (until about 30% of starting levels) in all the sublines studied, whatever the occurrence of metastasis, thus reinforcing the concept that the observed platelet drop in murine tumours is not related to metastatic growth. Moreover, in the same model, platelet drop could not be ascribed to intravascular platelet consumption, since it was observed both with sublines able to induce in vitro platelet aggregation and with those devoid of such property[18,20,21].

Some interest has been devoted to the blood platelet involvement during growth of human tumours implanted

Cambridge Books Online © Cambridge University Press, 2009

in athymic 'nude' mice. We studied the occurrence of hemostatic changes in mice implanted with several lines of human melanoma or colon carcinoma, but we did not find marked signs of platelet drop, in comparison with the above-mentioned changes in murine tumours[22].

In clinical conditions there is no consistent picture of platelet involvement associated with solid tumours and/or chemotherapy; patients with metastatic cancers may exhibit increased platelet activation as also indicated by enhancement of platelet turnover and decrease in platelet survival time[9]. These platelet abnormalities may be due to greater degree of destruction of lysosomal membrane fragments and to involvement of platelets in the low-grade intravascular activation of clotting detectable in the majority of cancer patients[4,7]. Increased factor VIII antigen, ristocetin cofactor and enhanced ADP-induced platelet aggregation have all been shown in vivo and reduced sensitivity of platelets to prostacyclin has also been reported. Patients with acute malignant disease may have elevated levels of ß-thromboglobulin and platelet factor 4[23]. Circulating activated platelets have also been demonstrated in cancer patients by detection of the platelet membrane antigens CD62 and CD 63[24]. More recently, a higher platelet count within the preoperative laboratory screening of patients with colorectal cancer has been reported in association with a poor prognosis[25].

Role of platelets in cancer growth and dissemination

Platelet–tumour cell interactions: in vitro studies

As already mentioned, these represent the vast majority of data available on the subject, since platelet interaction studies have been conducted either on tumour cell lines established in culture, or freshly harvested and disgregated from experimental tumours and surgical specimens of human tumours or on extracts prepared from malignant tissues.

Tumour cell-induced platelet blood aggregation

Tumour cells can induce platelet aggregation in vitro, as first described by Gasic et al.[26] and then by several other authors[8,9,27,28]. This phenomenon may be due either to shedding of microvesicles containing lipoproteins and sialic acid from tumour cells, or to generation of thrombin via the procoagulant activity of tumour cells or to leakage of ADP from the cells. The ability of tumour cells to induce in vitro platelet aggregation has been correlated with their invasiveness in vivo: a positive relation between the aggregating capacity and the metastatic potential has been proposed by several authors[18,29,30], while others have questioned this relationship[31,32]. Grignani and Jamieson observed that tumour cell ability to release ADP was irrelevant for tumour cell dissemination[32]. In contrast, Lampugnani and Crawford[33] found that highly metastatic tumours preferentially induced platelet aggregation through thrombin generation. Moreover, the presence of membrane glycoproteins immunologically related to the platelet glycoprotein (GP)IIb–IIIa on tumour cells has been associated with the tumour cell ability to induce platelet aggregation[34]. Further data indicate that α-thrombin activates the GPIIb–IIIa or $\alpha 2b\beta 3$ receptors on tumour cells and that this process mediates tumour cell-induced platelet aggregation and increases lung colony formation in mice[35,36].

Table 54.1. Platelet-released products possibly involved in promotion of cancer growth and dissemination

Growth factors	Cytokines and chemokines	Large adhesive proteins and receptors	Protease inhibitors
PDGF	PF-4	Fibrinogen	PAI-1/PAI-2
TGFß	ßTG	von Willebrand factor	α_2-antiplasmin
EGF	PBP	Fibronectin	TAFI
ECGF	IL-1	Thrombospondin	
HGF	VPF	P-selectin	
VEGF	Cytokine RANTES		
Others	Others		

Platelet-released products

Activated platelets release the components of their alpha granules, such as growth factors, cytokines, chemoattractants, adhesive proteins and proteolytic enzymes[9,37,38]. Some of these factors are involved in the metastatic process (see Table 54.1).

Two mitogenic factors were originally discovered in platelets: platelet-derived growth factor (PDGF) and transforming growth factor beta (TGFβ). Both factors promote cell migration, stimulate cell proliferation, and induce secretion of collagenase and of extracellular matrix proteins. Epidermal growth factor (EGF), endothelial cell growth factor (ECGF) and hepatocyte growth factor (HGF) were also found in platelets[9,37,38]. The latter factors seem to have more selected targets, such as the ability to promote angiogenesis.

Cambridge Books Online © Cambridge University Press, 2009

Platelets release IL-1[39] and the cytokine RANTES[40] from their alpha granules. IL-1 and other cytokines released from platelets modulate the activation of the endothelium and take part in malignant progression[41]. A vascular permeability growth factor (VPF) was also discovered in platelets[42].

Platelet factor 4 (PF4), β-thromboglobulin (β-TG) and platelet basic protein (PBP) are specific peptides, present in platelet α granules whose biological functions have recently been re-evaluated. PF4 was known as the most potent heparin neutralizing agent and as a powerful chemoattractant for fibroblasts, neutrophils and monocytes[43]. On the other hand, recombinant PF4 devoid of anti-heparin activity showed angiostatic effects and inhibitory activity in experimental models of mestastasis in mice[44]. PF4, β-TG and PBP are members of the family of cysteine-x-cysteine (c–x–c) chemokines or intercrines that are peptides with chemotactic properties[45]. Finally, platelets release large adhesive proteins, such as fibrinogen, von Willebrand factor, fibronectin and thrombospondin, whose functions will be discussed later in this chapter; platelets also release inhibitors of the fibrinolytic system, such as plasminogen activator inhibitor-1 (PAI-1) and alpha2-antiplasmin and other protease inhibitors possibly involved in tumour invasion and metastasis[37,46,47].

Platelet-mediated tumour cell adhesion and metastasis: revisited

Tumour cells show a selective organ preference to establish metastases[48], but the mechanisms underlying this selection are largely unknown. The adhesion of tumour cells to the vessel wall is stimulated by membrane antigens present on activated platelets[49,50]. Bastida et al.[51] found that tumour cell adhesion induced by platelets was increased by high shear forces and inhibited by peptides active as competitors of cell adhesion to fibronectin.

Platelets and leukocytes can mediate the adhesion of tumour cells to the endothelium and to the extracellular matrix through the activation of specific adhesive receptors and the release of cytokines and chemokines[52]. Inflammatory cytokines would activate endothelial cells to a proinflammatory and a procoagulant state, thus facilitating tumour cell adhesion[53,54]. IL-1 seems to play a special role in this process, since it stimulates procoagulant activity, synthesis of PAI-1, release of prostacyclin and exposure of adhesive receptors on endothelial cells[53]. This cytokine, similarly to TNF, can exert a prometastatic effect in human and murine tumour models, as shown by Bani et al.[55]. Such an effect could be ascribed to IL-1-induced enhancement of adhesive interactions between tumour cells and the vascular wall[56].

Activated platelets, as mentioned, release large adhesive proteins, such as fibrinogen, von Willebrand factor, thrombospondin, fibronectin and P-selectin[38]. Fibrinogen and von Willebrand factor have a physiological role for platelet aggregation, adhesion and activation. Their involvement in the process of tumour dissemination is secondary to the interaction of tumour cells, platelets and endothelium with specific receptors, which will be discussed later on. Very recently, the role of fibrinogen as a determinant of the metastatic potential of circulating tumour cells has been re-evaluated with the help of genetically deficient models[57]. Rather than merely representing an important component of the tumour stroma, fibrinogen was found to be crucial to sustain adhesion and survival of tumour cells within the lungs with mechanisms not completely understood. Fibrinogen is known to support cell–cell adhesion through integrin and non-integrin receptors. The hypothesis that soluble fibrinogen and platelets contribute significantly to the exit of tumour cells from the vasculature is consistent with findings that fibrinogen and platelet-mediated adhesion may be important in stable leukocyte adhesion and transendothelial migration[58]. Thrombospondin (TSP), a protein originally discovered in platelets, is endowed with the ability to stimulate adhesion, migration and proliferation of normal and tumour cells[59]. This large adhesive protein seems to play a role in tumour dissemination, as suggested by the observation that TSP enhances the adhesion of human melanoma cells[60] and increases the number of pulmonary metastases, when injected together with B16 melanoma cells in mice[61]. Synthetic peptides mimicking the COOH terminal of TSP inhibit platelet aggregation, compete for tumour cell adhesion to substrates and reduce artificial metastases in experimental tumour models[62].

The requirement for platelets to adhere to tumour cells, in order to elicit a prometastatic potential was recognized several years ago[63] and the role of specific adhesive receptors as molecular bridges of this interaction has been defined.

The role of αIIbβ3

In their original report Karpatkin et al.[63] were able to reduce in vivo pulmonary metastasis of a colon carcinoma cell line and in vitro tumour cell-platelet binding by monoclonal antibody blockade of platelet αIIbβ3. In this study a polyclonal anti-von Willebrand antibody also reduced metastatization, suggesting that von Willebrand factor, binding αIIbβ3, bridges tumour cells and platelets. In agreement with this result, platelet interaction with melanoma cells was prevented by a monoclonal antibody against the platelet integrin[64]. Tumour cells may actually themselves express αIIbβ3-like complexes; αIIbβ3 receptors have been found

Cambridge Books Online © Cambridge University Press, 2009

in different types of human and animal tumours[34,65,66,67]. The presence of αIIbβ3 appeared to be positively related with the platelet aggregating ability in a series of B16 melanoma cell lines with different metastatic activity[28]. $\alpha_V\beta$3 is also present in melanoma cells where it appeared to be a marker of malignancy, since it was associated with invasive and metastatic behaviour[68]. Most notably, when the expression of the β3 subunit was examined in benign and malignant lesions of human melanocytes, it was found to be restricted to malignant lesions in vertical growth phase and in metastatic melanomas, suggesting that the presence of this integrin may be important in the development of tumour invasiveness[69]. In vitro experiments showed that $\alpha_V\beta$3 in melanoma cells mediates melanoma cell attachment to activated platelets under flow in a platelet αIIbβ3-dependent manner[70,71]. In addition to melanoma, in other malignancies, including glioma[72], and ovarian and breast cancer[73,74], expression of αVβ3 in tumour cells correlates with tumour progression.

In circulating normal cells, such as platelets and leukocytes, integrins are in an inactive state. They acquire the ability to bind the ligand and to sustain a shear resistant cell adhesion to the vascular wall as a consequence of cell activation by extracellular stimuli. As for other integrins, the adhesive function of αIIbβ3 as well as $\alpha_V\beta$3 is functionally regulated by the cell[75]. This important aspect of integrin biology may also be relevant in the process of tumour cell adhesion and invasion. Using a monoclonal antibody specifically binding an activation epitope of αIIbβ3, it was found that metastatic melanoma cells express the integrin in the high affinity state[76].

More recently Felding-Habermann et al.[77] showed that not only activated $\alpha_V\beta$3 was expressed by breast cancer cells isolated from human metastasis, but also that only cells expressing the activated state of $\alpha_V\beta$3 were able to arrest on platelets, during blood flow in vitro, and promoted metastasis in a mouse model in vivo. These observations strengthen the hypothesis that alterations in the control of integrin (particularly β3) activation within the tumour cells, may influence their metastatic potential.

The role of selectins

Studies on the mechanism of entry of circulating leukocytes into tissues during inflammation showed that this is a multistep process initiated by attachment and rolling of leukocytes on the vessel wall mediated by selectins. P-, E- and L-selectins are typical type I proteins composed of different domains. These include an amino terminal C-type lectin domain, a single epidermal growth factor (EGF)-like domain, from two to nine short consensus repeat (SCR) domains, a single membrane spanning region, and a cytoplasmic tail[78]. The selectins bind carbohydrate structures containing the sialyl-Lewis X antigen appropriately expressed by specific proteins on the cell surface[79]. Different candidate glycoprotein ligands for each of the selectins have been identified. The most extensively characterized selectin ligand is the recently cloned P-selectin glycoprotein ligand-1 (PSGL-1), a mucin-like protein with a unique structure formed by a disulfide linked homodimer of two identical 120 kD chains. The first studies showed that PSGL-1 is mainly expressed on leukocytes. Recently, analysis of murine tissues indicates that the expression of PSGL-1 is widespread. However, precisely which cells, apart from leukocytes, express PSGL-1 and what is the role of PSGL-1 is on these cells, is presently unknown in humans and is an important area of future investigations[80]. Among the carcinoma associated glycans, the expression of sialyl Lewis^X and sialyl Lewis^a correlates with tumour progression, metastatic spread, and poor prognosis[81–89]. On the other hand, treatment of tumour cells with benzyl-a GalNac to suppress the *o*-linked carbohydrate chain extension resulted in a diminished expression of sLeX and sLea on these tumour cells and reduced tumour cell adhesion to activated endothelial cells or platelets[90].

Different tumour cell lines are able to bind selectins via not yet identified receptor(s) containing the sialyl-Lewis X antigen. Colon carcinoma cell lines bind to cytokine-activated endothelial cells through E-selectin[91], and leukocytes and platelets through L- and P selectin binding mucins[92,93] distinct from PSGL-1[94]. Similarly, neuroblastoma and small cell lung cancer cells adhere to activated platelets through P-selectin[95]. Human small cell lung carcinoma cells and breast carcinoma cells are also negative for PSGL-1 and bind P-selectin through CD24, a mucin-type glycosylphosphatidylinositol-linked protein[96]. The role of platelet P-selectin in tumour metastasis is also strongly supported by the experiments of Kim and coworkers[97]. These authors showed that human colon carcinoma cells, implanted subcutaneously, grow at a lower rate and generate fewer lung metastases when injected intravenously in P-selectin deficient as compared with control mice. This effect correlated with reduced formation of tumour cell–platelet clumps in the lung vasculature of P-selectin knock-out mice[97].

More recently, CD 24 was suggested to mediate the lung metastatization by adenocarcinoma cells via a P-selectin dependent mechanism[98]. Investigating the adhesive mechanism of three human colon carcinoma cell lines on adherent platelets in flow conditions McCarty et al.[99] showed that the sequential engagement of P-selectin and aIIbβ3 is a requisite for optimal adhesion of two of them; the cells of the third cell line did not interact with P-selectin

Cambridge Books Online © Cambridge University Press, 2009

and were not recruited on platelets under flow despite the fact that they adhered on platelets under static conditions. Also in this case P-selectin ligand was distinct from PSGL-1 and CD24. Altogether, these studies indicate that during the process of metastatization tumour cells recapitulate the adhesion cascade, used by normal circulating cells, namely platelets or leukocytes, to accomplish normal functions such as hemostasis and immune responses. Figure 54.1 outlines schematically a proposed mechanism for these cellular interactions where two processes are considered: (i) the formation of platelet–tumour cell microemboli; (ii) the recruitment of tumour cells at sites of vascular damage (Fig. 54.1).

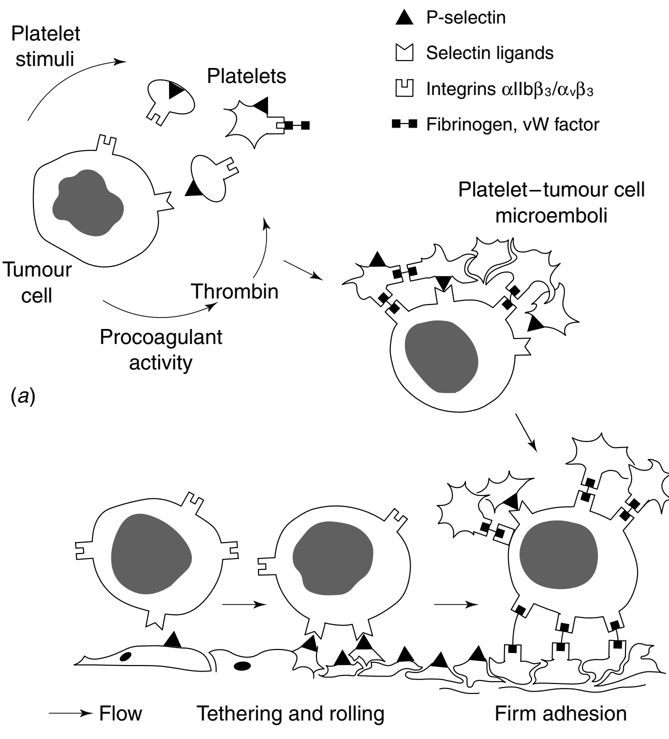


Fig. 54.1. Schematic representation of platelet–tumour cell emboli formation and of platelet-mediated tumour cell recruitment at sites of vascular damage. (*a*) Platelets activated by tumour cell-derived stimuli adhere to the tumour cell via P-selectin and β_3 integrins and forming microemboli assist tumour cell arrest and metastatization. (*b*) Platelets activated at a site of vascular damage mediate tumour cell recruitment through P-selectin-dependent tethering and rolling and β_3 integrin-dependent firm adhesion.

Platelets and angiogenesis

Although described some decades ago, the process of new vessel formation within the tumour and at the tumour host/interface, i.e. the so-called angiogenesis, has attracted increasing interest in recent years, due to the identification of some endogenous active factors (see angiostatin and endostatin) and to the preclinical and preliminary clinical tests on these agents for anticancer treatment. These agents would be active by depriving the tumour of the necessary blood supply through blockade of new vessel formation at the tumour site[100].

Among endogenous mediators known to influence the angiogenetic process, vascular endothelial growth factor (VEGF) is a potent agonist of new vessel formation. Blood platelets have been found to release VEGF upon activation and to act as major transporters of VEGF in the human circulation[101]. The hypothesis has been put forward that a specific tumour cell/ endothelium/platelet interaction can contribute to tumour-induced angiogenesis, since platelets activated f.i. by thrombin (evolved from tumour procoagulants) release VEGF. VEGF, in turn, can induce endothelial cells to release von Willebrand Factor, which may contribute to further recruitment of platelets by stimulating their adhesive properties[102].

Presumably, the increased platelet-binding capacity of the tumour vasculature and the subsequent activation of platelets are regulated by stimuli of the tumour cell and may differ for each tumour type.

Among mediators released from blood platelets during clotting, sphingosine 1-phosphate (S1P) is a potent, specific and selective endothelial cell chemoattractant that accounts for most of the strong endothelial cell chemotactic activity of blood serum. S1P would act by activating a receptor-dependent process[103]. The putative role of platelets in tumour angiogenesis is presently the topic of investigations at the clinical level in different series of patients with solid tumours.

Platelets and host's immune response to the tumour

The contribution of blood platelets to cancer metastasis depends not only on their interactions with the biological barrier represented by the leukocyte/endothelial cell system in the phase of extravasation, but may also be ascribed, at least in some tumours, to protection by platelets from tumour cell killing by natural killer (NK) cells. It has been shown that such protection occurs both in vitro and in vivo. In three different tumour cell lines from a mouse model of experimental metastasis, tumour seeding in the target organs was reduced when the host was platelet depleted, but only if the tumour cells were NK sensitive[104]. Aggregation of platelets around tumour cells also inhibited in vitro the tumourilytic activity by NK cells; besides providing a physical barrier and a steric hindrance between tumour cells and NK cells, platelets may also interfere with recognition for killing of tumour cells by NK

Cambridge Books Online © Cambridge University Press, 2009

cells. However, since platelet-caused NK inhibition was independent of the platelet donor haplotype and was equally observed with platelets from β2-microglobulin-deficient mice, a MHC class 1-dependent inhibitory mechanism is unlikely[104]. Alternatively, platelets would not only shield tumour cells but also inhibit cytolytic function of NK by some other mechanism (release of tumour cell protective factors). The presence of a platelet 'cloak' around tumour cells would also protect the latter from a closer interaction with monocytes involved physiologically in elimination of metastatic tumour cells during the early phase of metastasis. In systems of mucin-producing human tumour cells growing in athymic mice, the inhibition of platelet/tumour cell aggregation by heparin or by P selectin-deficiency leads to reduced metastasis growth; this antimetastatic effect was also concomitant with an increased association of MAC-1 positive monocytes with tumour cells[105].

Platelet–tumour cell interactions: in vivo studies

The platelet–tumour cell interactions discussed so far, mainly in in vitro systems, offer altogether biological plausibility for a role of platelets in tumour growth and dissemination, but only from in vivo studies can direct proof be obtained on the platelet involvement in tumours. For several years, experimental models where the host's hemostatic system had been pharmacologically modified, have been followed for tumour and metastasis growth[18]. This approach has been more recently integrated with the use of genetically modified animals, selectively lacking one or more factors of the hemostatic system[6].

Pharmacological implications

We shall now discuss some of the pharmacological approaches used with the general 'caveat' that none of these has so far found a well-established clinical counterpart. At the moment there are few studies of anticoagulants/antiplatelet drugs which really fulfil modern criteria of controlled randomized trials to evaluate drug efficacy.

The published studies have different designs (case reports, phase I, II and III studies, and largely retrospective epidemiological reviews) and most studies have tested anticoagulants in combination with cytotoxic drugs which makes evaluation of the results more difficult. From the data obtained so far, the following conclusions can be drawn for clinical studies:

- for small cell lung carcinoma, therapy with warfarin may lead to a longer period of disease-free survival and overall survival;
- therapy with RA-233, a dipyridamole analogue, leads to an increased overall survival in patients with localized non-small cell lung carcinoma;
- the efficacy of low-dose acetylsalicylic acid in colorectal cancer is still the subject of controversy. Treatment with sulindac, another non-steroidal anti-inflammatory drug, leads to a statistically significant reduction in amount and size of colon polyps in adenomatosis coli. It is noteworthy that not only the efficacy but also the mechanism of action of these anti-inflammatory agents in colorectal cancer is still debated and the role of platelets uncertain.

In experimental models the pioneering studies of Gasic and Gasic[11], and Gasic et al.[12] showed that induction of thrombocytopenia by antiplatelet serum or by treatment with neuraminidase inhibited metastases in experimental animal models. The effect of platelet antiserum was confirmed by Pearlstein et al.[106] and also by our group[9]. The intravenous injection of B16BL6 melanoma in mice, pretreated with antiplatelet serum, causes a 70% reduction in lung colony formation after 15 days. It is likely that the antimetastatic effect exerted by the antiplatelet serum may be the consequence of concomitant alterations of the endothelium, due to the removal of the sialic acid coating, which seems to be important for tumour dissemination, at least in conditions of high shear stress[90].

Various antiplatelet drugs have been used in the attempt to inhibit metastases[8,28,107]. The antimetastatic effect of aspirin was observed by Gasic et al.[13] but not confirmed by other investigators[108]. Ticlopidine, another potent platelet aggregation inhibitor, was unable to reduce metastasis in a model of spontaneous dissemination[63]. The effects of prostacyclin[109,110], prostacyclin analogues[111] and thromboxane synthetase inhibitors[112,113] were rather controversial. Calcium channel blockers have successfully been used as antimetastatic agents[111]. Dipyridamole and the pyrimido-pyrimidine derivative RA233 were active in some experimental models.

Thus, the effect of antiplatelet agents on metastasis was often controversial[28]. This may be the consequence of heterogeneity of tumours and administration schedules or may reflect the fact that antiplatelet drugs have multiple effects on different cell targets (anti-inflammatory, antiadhesive, immunomodulatory, besides platelet inhibition). As a general comment, antiplatelet agents have been found to be much more active in preventing lung colony formation ('artificial metastasis') than the 'spontaneous' dissemination process. This is a type of behaviour shared by virtually all anticoagulant, antiplatelet and defibrinating agents used so far; it suggests that, in the artificial model, whatever is pharmacologically operated prior to tumour cell injection in order to reduce blood coagulability and/or

Cambridge Books Online © Cambridge University Press, 2009

blood cell stickiness between them and to the vascular wall, the result will be a longer circulation time of tumour cells in the host and a higher rate of their destruction by the reticuloendothelial system: as a result, metastatic growth will be reduced.

In the last 10–15 years some platelet–tumour cell interactions have been more deeply studied in their multicellular scenario (see the section on cell adhesion).

The in vitro studies have also provided new candidates for in vivo testing against cancer metastasis; more selective agents, whose effects are directed towards specific cell functions, have recently been developed.

Monoclonal antibodies to αIIbβ3

The monoclonal antibody (mAb) 7E3, a specific inhibitor of the platelet αIIbβ3 receptor, was rather active as an inhibitor of human platelet aggregation[116]. This antibody reduced lung colony formation after intravenous injection of tumour cells in mice[63]. Monoclonal antibodies to αIIbβ3 also inhibited the adhesion of human tumour cells to fibronectin[117] and tumour cell induced platelet aggregation[34]. In addition, Boukerche et al.[64] observed a reduction of the growth of human melanoma cells in nude mice after treatment with LYP18, another mAb to αIIbβ3.

RGD peptides

Renewed interest in selective inhibition of platelet–tumour cell interaction was given by synthetic peptides with RGD sequence[118]. The intravenous injection of B16 melanoma cells together with peptide gly–arg–gly–asp–ser (GRGDS), significantly inhibited lung colony formation in mice[119]. This effect was attributed to accelerated disappearance of tumour cells from the lungs of treated mice. Modified peptides, lacking the RGD sequence, such as gly–arg–gly–glu–ser (GRGES) were not active[120]. Several RGD-containing peptides have been used successfully in experimental models of tumour dissemination[121–123]. However, the short half-life and the reversibility of the effect of these agents on platelets have limited their use as antimetastatic agents. The effect of RGD-containing peptides on metastasis may be due to the blockade of platelet–tumour cell interactions or to the inhibition of tumour cell adhesion to fibronectin. To clarify this problem, we studied arg–gly–asp–trp (RGDW),a tetrapeptide with a potent inhibitory effect on platelet aggregation and a more limited effect on cell adhesion, in the B16BL6 lung colony assay.RGDW was about ten times more active in inhibiting ADP-induced platelet aggregation and ^{125}I-fibrinogen binding to platelets, as compared to arg–gly–asp–ser[124]. RGDW was also more potent than RGDS in inhibiting B16BL6 lung colony formation[125]. Arg–gly–gly–trp(RGGW),a tetrapeptide devoid of antiplatelet activity, was also inactive on metastases, suggesting that inhibition of platelet functions was crucial, at least in our model.

Disintegrins

Disintegrins are a class of snake venom-derived peptides, characterized by the presence of the RGD sequence and a cysteine-rich region. Disintegrins are several orders of magnitude more potent than small RGD peptides as inhibitors of platelet aggregation and cell adhesion[126,127]. Trigramin, a peptide purified from the venom of *Trimeresurus gramineus*, showed a potent inhibitory effect on the adhesion of human melanoma cells to fibronectin and fibrinogen[128]. Batroxostatin also caused a potent inhibition of the adhesion of human and murine melanoma cells to fibronectin[129] and triflavin inhibited the adhesion of B16 melanoma cells to various substrates[130]. We found that albolabrin, a disintegrin derived from the venom of *Trimeresurus albolabris*, was about 2000-fold more active than RGDS at inhibiting mouse platelet aggregation (IC_{50}: 166 nM and 50 μM, respectively) and was also more active than RGDS at protecting mice from pulmonary thromboembolism[131]. Similarly, albolabrin was more active than RGDS at inhibiting B16BL6 melanoma lung colony formation in mice[132]. Since albolabrin prevented the accumulation of ^{51}Cr-labelled platelets in the lungs of treated mice, it is possible that its antimetastatic effect may be due to the diversion of platelet–tumour cell emboli from the lungs of treated mice. Eristostatin, a disintegrin with a more selective inhibitory effect on platelet aggregation, was also found to have more potent inhibitory effects on lung colony formation than albolabrin (IC_{50}: 0.05 μM and 1.0 μM, respectively)[133]. Analysis of disintegrin structure revealed that disintegrins with the RGDW sequence, such as eristostatin, have a higher affinity for αIIbβ3 receptors than other RGDX sequence, thus explaining their increased activity on platelet functions[134]. The design of even more selective inhibitors of cell functions, such as platelet aggregation or cell adhesion, may improve the effectiveness of these agents on metastases.

Conclusions

Platelet interactions with tumour cells have been described both in vitro and in vivo. Platelet activation can stimulate tumour cell adhesion to the vessel wall, with a mechanism involving selectins, integrins, IgG-like molecules and other adhesive receptors. Similar receptors present on neutrophils, monocytes and endothelial cells take part in this process, with a concerted action. Growth

Cambridge Books Online © Cambridge University Press, 2009

factors, cytokines, chemokines and other products released after platelet aggregation and activation may further stimulate the adhesion of tumour cells to activated endothelium. More experimental work is needed to translate knowledge on this fascinating machinery into new pharmacological tools for cancer and mestastasis control; new approaches aimed at inhibiting receptor-mediated platelet adhesion and activation may be promising for the prevention and cure of metastasis.

Acknowledgements

The authors' work reviewed in this chapter has been supported by the Italian Ministry of Health (Rome, Italy), the Italian National Research Council (CNR, Rome, Italy) and the Segré Foundation (Geneva, Switzerland). The authors wish to thank Drs Andreina Poggi and Cosmo Rossi for long-lasting active collaboration in the field of 'hemostasis and tumours'.

Rosanna Tucci, Alexandra Cianci and the G. A. Pfeiffer Memorial Library staff helped to prepare this manuscript.

References

1 Trousseau A. Phlegmasia alba dolens. In *Clinique medicale de l'Hotel Dieu de Paris*, 2nd edn. Paris: Baillière. 1865; 3: 654–712.
2 Billroth T. *Lectures on surgical pathology and therapeutics: a handbook for students and practitioners.* 8th edn. London: The New Sydenham Society. 1878; 1877–1878.
3 Poste G, Fidler JJ. The pathogenesis of cancer metastasis. *Nature* 1980; 283: 139–146.
4 Donati MB, Poggi A. Malignancy and haemostasis. *Br J Hematol* 1980; 44: 173–182.
5 Donati MB, Semeraro N. Cancer cell procoagulants and their pharmacological modulation. *Haemostasis* 1984; 14: 422–429.
6 Donati MB. Cancer and thrombosis: from phlegmasia alba dolens to transgenic mice. *Thromb Haemost* 1995; 74: 278–281.
7 Donati MB, Falanga A. Thrombosis and malignancy: pathogenetic mechanisms. *Acta Haematol* 2001; 106: 18–24.
8 Poggi A, Donati MB. Platelets and tumor metastasis. In Page CP, ed. *The platelet in health and disease.* Oxford: Blackwell Scientific Publications. 1991; 175–190.
9 Poggi A, Rossi C, Beviglia L, Calabrese R, Donati MB. Platelets–tumour cell interaction. In Joseph M, ed. *The handbook of immunopharmacology – immunopharmacology of platelets.* London: Academic Press. 1995; 151–165.
10 Wood S. Pathogenesis of metastasis formation observed in vivo in the rabbit ear chamber. *AMA Arch Pathol* 1958; 66: 550–568.
11 Gasic G, Gasic T. Removal of sialic acid from the cell coat in tumor cells and vascular endothelium and its effects on metastasis. *Proc Natl Acad Sci USA* 1962; 48: 1172–1177.
12 Gasic GJ, Gasic TB, Stewart GC. Antimetastatic effects associated with platelet reduction. *Proc Natl Acad Sci USA* 1968; 61: 46–52.
13 Gasic GJ, Gasic TB, Murphy S. Antimetastatic effect of aspirin. *Lancet* 1972; ii: 932–933.
14 Cerletti C, Evangelista V, de Gaetano G. P-selectin-$\beta 2$ integrin cross-talk: a molecular mechanism for polymorphonuclear leukocyte recruitment at the site of vascular damage. *Thromb Haemost* 1999; 82: 787–793.
15 Poggi A, Donati MB, Garattini S. Fibrin and cancer cell growth: problems in the evaluation of experimental models. In *Malignancy and the hemostatic system.* New York: Raven Press. 1981: 89–101.
16 Poggi A, Polentarutti N, Donati MB, de Gaetano G, Garattini S. Blood coagulation changes in mice bearing Lewis lung carcinoma, a metastasizing tumor. *Cancer Res* 1977; 37: 272–277.
17 Mussoni L, Poggi A, de Gaetano G, Donati MB. Effect of ditazole, an inhibitor of platelet aggregation, on a metastasizing tumor in mice. *Br J Cancer* 1978; 37: 126–129.
18 Donati MB, Rotilio D, Delaini F, Giavazzi R, Mantovani A, Poggi A. Animal models for the study of platelet tumor cell interactions. In Jamieson GA, ed. *Interaction of platelets and tumor cells.* New York: Alain Liss. 1982: 159–174.
19 Chmielewska J, Poggi A, Mussoni L, Donati MB, Garattini S. Blood coagulation changes in JW sarcoma, a new metastasizing tumor in mice. *Eur J Cancer* 1980; 16: 1399–1407.
20 Delaini F, Giavazzi R, De Bellis Vitti G, Alessandri G, Mantovani A, Donati MB. Tumor sublines with different metastatic capacity induce similar blood coagulation changes in the host. *Br J Cancer* 1981; 43: 100–104.
21 Vicenzi E, Lampugnani MG, Bolognese Dalessandro AP, Niewiarowska A, de Gaetano G, Donati MB. Dissociation between thromboxane generation and metastatic potential in cells from a murine fibrosarcoma. Studies with a selective thromboxane synthase inhibitor. *Int J Cancer* 1987; 39: 488–491.
22 Bani MR, Falanga A, Alessio MG et al. Blood coagulation changes in nude mice bearing human colon carcinomas. *Int J Cancer* 1992; 50: 75–79.
23 Al-Mondhiry H. Beta-thromboglobulin and platelet factor 4 in patients with cancer: correlation with the stage of disease and the effect of chemotherapy. *Am J Hematol* 1983; 14: 105–111.
24 Wehmeier A, Tschope D, Esser J, Menzel C, Nieuwenhuis HK, Schneider W. Circulating activated platelets in myeloproliferative disorders. *Thromb Res* 1991; 61: 271–278.
25 Monreal M, Fernandez-Llamazares J, Pinol M et al. Platelet count and survival in patients with colorectal cancer – a preliminary study. *Thromb Haemost* 1998; 79: 916–918.
26 Gasic GJ, Gasic TB, Galanti N, Johnson T, Murphy S. Platelet tumor cell interactions in mice: the role of platelets in the spread of malignant disease. *Int J Cancer* 1973; 11: 704–718.

Cambridge Books Online © Cambridge University Press, 2009

27 Jamieson GA, Bastida E, Ordinas A. Interaction of platelets and tumor cells. In Macintyre DE, Gordon JL, eds. *Platelets in biology and pathology*. Amsterdam: Elsevier Science Publisher. 1987; 3: 161–189.
28 Honn KB, Tang DG, Crissman JD. Platelets and cancer metastasis: a casual relationship? *Cancer Metastasis Rev* 1992; 11: 325–351.
29 Pearlstein E, Salk PL, Jogieeswaran G, Karpatkin S. Correlation between spontaneous metastatic potential, platelet aggregating activity of cell surface extracts, and cell surface sialylation in 10 metastatic variant derivatives of a rat renal sarcoma cell line. *Proc Natl Acad Sci USA* 1980; 77: 4336–4339.
30 Sugimoto Y, Oh-hara T, Watanabe M, Saito H, Yamori T, Tsuruo T. Acquisition of metastatic ability in hybridomas between two low metastatic clones of murine colon adenocarcinoma 26 defective in either platelet aggregating activity or in vivo growth potential. *Cancer Res* 1987; 47: 4396–4401.
31 Estrada J, Nicolson GL. Tumor–cell platelet aggregation does not correlate with metastatic potential of rat 13762NF mammary adenocarcinoma tumor cell clones. *Int J Cancer* 1984; 34: 101–105.
32 Grignani G, Jamieson GA. Platelets in tumor metastasis: generation of adenosine diphosphate by tumor cells is specific but unrelated to metastatic potential. *Blood* 1988; 71: 844–849.
33 Lampugnani MG, Crawford N. Tumor cell induced platelet aggregation: studies with cloned metastatic and non-metastatic variants. *Invasion Metastasis* 1987; 7: 311–324.
34 Chopra H, Hatfield JS, Chang YS et al. Role of tumor cytoskeleton and membrane glycoprotein IRGpIIb/IIIa in platelet adhesion to tumor cell membrane and tumor cell-induced platelet aggregation. *Cancer Res* 1988; 48: 3787–3800.
35 Nierodzik ML, Plotkin A, Kajumo F, Karpatkin S. Thrombin stimulates tumor–platelet adhesion in vitro and metastasis in vivo. *J Clin Invest* 1991; 87: 229–236.
36 Wojtukiewicz MZ, Tang DG, Nelson KK, Walz DA, Diglio CA, Honn KV. Thrombin enhances tumor cell adhesive and metastatic properties via increased alpha IIb beta 3 expression on the cell surface. *Thromb Res* 1992; 68: 233–245.
37 Niewiarowski S, Holte JC. Biochemistry and physiology of secreted platelet proteins. In Colman RW, Hirsh J, Marder VJ, Salzman EW, eds. *Haemostasis and thrombosis*. Philadelphia: Lippincott. 1987: 618–630.
38 Poggi A, Stella M, Donati MB. The importance of blood cell–vessel wall interactions in tumor metastasis. *Baillières Clin Haematol* 1993; 6: 731–752.
39 Hawrylowicz CM, Howels GL, Feldman M. Platelet-derived interleukin 1 induces human endothelial adhesion molecule expression and cytokine production. *J Exp Med* 1991; 174: 785–790.
40 Kameyoshe Y, Dorschner A, Mallet AI, Cristophers E, Schroder JM. Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. *J Exp Med* 1992; 176: 587–592.
41 Hawrylowicz CM. Viewpoint: a potential role for platelet derived cytokines in the inflammatory response. *Platelets* 1993; 4: 1–10.
42 Dvorak HF, Nagy JA, Dvorak AM. Structure of solid tumors and their vasculature: implications for therapy with monoclonal antibodies. *Cancer Cells* 1991; 3: 77–85.
43 Deuel TF, Senior RM, Huang JS, Griffin GL. Chemotaxis of monocytes and neutrophils to platelet derived growth factor. *J Clin Invest* 1982; 69: 1046–1049.
44 Maione TE, Gray GS, Hunt AJ, Sharpe RJ. Inhibition of tumor growth in mice by an analogue of platelet factor 4 that lacks affinity for heparin and retains potent angiostatic activity. *Cancer Res* 1991; 51: 2077–2083.
45 Brandt E, Flad H-D. Structure and function of platelet-derived cytokines of the B-thromboglobulin/interleukin 8 family. *Platelets* 1992; 3: 295–305.
46 Herlyn M, Malkowicz SB. Regulatory pathways in tumor growth and invasion. *Lab Invest* 1991; 65: 262–271.
47 Duffy MJ. The role of proteolytic enzymes in cancer invasion and metastasis. *Clin Exp Metastasis* 1992; 10: 145–155.
48 Nicolson GL. Tumor and host molecules important in the organ preference of metastasis. *Semin Cancer Biol* 1991; 2: 143–154.
49 Menter DG, Steinert BW, Sloane BF et al. Role of platelet membrane in enhancement of tumor cell adhesion to endothelial cell extracellular matrix. *Cancer Res* 1987; 47: 6751–6762.
50 Abecassis J, Million-Collard R, Klein-Soyer C et al. Adhesion of human breast cancer cell line MCF-7 to human vascular endothelial cells in culture. Enhancement by activated platelets. *Int J Cancer* 1987; 40: 525–531.
51 Bastida E, Almirall L, Bertomeu MC, Ordinas A. Influence of shear stress on tumor–cell adhesion to endothelial-cell extracellular matrix and its modulation by fibronectin. *Int J Cancer* 1989; 43: 1174–1178.
52 Roth GJ. Platelets and blood vessels: the adhesion event. *Immunol Today* 1992; 13: 100–105.
53 Mantovani A, Bussolino F, Dejana E. Cytokine regulation of endothelial cell function. *FASEB J* 1992; 6: 2591–2599.
54 Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* 2001; 357: 539–545.
55 Bani MR, Garofalo A, Scanziani E, Giavazzi R. Effect of interleukin-1-beta on metastasis formation in different tumor systems. *J Natl Cancer Inst* 1991; 83: 119–123.
56 Giavazzi R, Chirivi RG, Garofalo A, Rambaldi A, Hemingway T, Pigott R, Gearing AJ. Soluble intercellular adhesion molecule 1 is released by human melanoma cells and is associated with tumor growth in nude mice. *Cancer Res* 1992; 52: 2628–2630.
57 Palumbo JS, Kombrinck KW, Drew AF et al. Fibrinogen is an important determinant of the metastatic potential of circulating tumor cells. *Blood* 2000; 96: 3302–3309.
58 Languino LR, Duperray A, Joganic KJ, Fornaro M, Thornton GB, Altieri DC. Regulation of leukocyte–endothelium interaction and leukocyte transendothelial migration by intercellular adhesion molecule 1-fibrinogen recognition. *Proc Natl Acad Sci USA* 1995; 92: 1505–1509.

Cambridge Books Online © Cambridge University Press, 2009

59 Frazier WA. Thrombospondin: a modular adhesive glycoprotein of platelets and nucleated cells. *J Cell Biol* 1987; 105: 625–632.

60 Tuszynski GP, Rothman VL, Murphy A, Siegler K, Smith L, Smith S, Karczewski J, Knudsen KA. Thrombospondin promotes cell–substratum adhesion. *Science* 1987; 236: 1570–1573.

61 Tuszynski GP, Gasic TB, Rothman VL, Knudsen KA, Gasic GJ. Thrombospondin, a potentiator of tumor cell metastasis. *Cancer Res* 1987; 47: 4130–4133.

62 Tuszynski GP, Rothman VL, Deutch AH, Hamilton BK, Eyal J. Biological activities of peptides and peptide analogues derived from common sequences present in thrombospondin, properdin, and malarial proteins. *J Cell Biol* 1992; 116: 209–217.

63 Karpatkin S, Pearlstein E, Ambrogio C, Coller BS. Role of adhesive proteins in platelet tumor interaction in vitro and metastasis formation in vivo. *J Clin Invest* 1988; 81: 1012–1019.

64 Boukerche H, Berthier-Vergnes O, Tabone E, Dore JF, Leung LL, McGregor JL. Platelet–melanoma cell interaction is mediated by the glycoprotein IIb–IIIa complex. *Blood* 1989; 74: 658–663.

65 Boukerche H, Berthier-Vergnes O, Bailly M, Dore JF, Leung LL, McGregor JL. A monoclonal antibody (LYP18) directed against the blood platelet glycoprotein IIb/IIIa complex inhibits human melanoma growth in vivo. *Blood* 1989; 74: 909–912.

66 Chen YQ, Gao X, Timar J et al. Identification of the alpha IIb beta 3 integrin in murine tumor cells. *J Biol Chem* 1992; 267: 17314–17320.

67 Oleksowicz L, Mrowiec Z, Schwartz E, Khorshidi M, Dutcher JP, Puszkin E. Characterization of tumor-induced platelet aggregation: the role of immunorelated GPIb and GPIIb/IIIa expression by MCF-7 breast cancer cells. *Thromb Res* 1995; 79: 261–274.

68 Felding-Habermann B, Ruggeri ZM, Cheresh DA. Distinct biological consequences of integrin alpha v beta 3-mediated melanoma cell adhesion to fibrinogen and its plasmic fragments. *J Biol Chem* 1992; 267: 5070–5077.

69 Albelda SM, Mette SA, Elder DE, Stewart R, Damjanovich L, Herlyn M, Buck CA. Integrin distribution in malignant melanoma: association of the beta 3 subunit with tumor progression. *Cancer Res* 1990; 50: 6757–6764.

70 Felding-Habermann B, Habermann R, Saldivar E, Ruggeri ZM. Role of beta3 integrins in melanoma cell adhesion to activated platelets under flow. *J Biol Chem* 1996; 271: 5892–5900.

71 Dardik R, Kaufmann Y, Savion N, Rosenberg N, Shenkman B, Varon D. Platelets mediate tumor cell adhesion to the subendothelium under flow conditions: involvement of platelet GPIIb–IIIa and tumor cell alpha(v) integrins. *Int J Cancer* 1997; 70: 201–207.

72 Gingras MC, Roussel E, Bruner JM, Branch CD, Moser RP. Comparison of cell adhesion molecule expression between glioblastoma multiforme and autologous normal brain tissue. *J Neuroimmunol* 1995; 57: 143–153.

73 Pignatelli M, Cardillo MR, Hanby A, Stamp GW. Integrins and their accessory adhesion molecules in mammary carcinomas: loss of polarization in poorly differentiated tumors. *Hum Pathol* 1992; 23: 1159–1166.

74 Liapis H, Flath A, Kitazawa S. Integrin alpha V beta 3 expression by bone-residing breast cancer metastases. *Diagn Mol Pathol* 1996; 5: 127–135.

75 Shattil SJ, Kashiwagi H, Pampori N. Integrin signaling: the platelet paradigm. *Blood* 1998; 91: 2645–2657.

76 Timar J, Trikha M, Szekeres K, Bazaz R, Honn K. Expression and function of the high affinity alphaIIbbeta3 integrin in murine melanoma cells. *Clin Exp Metastasis* 1998; 16: 437–445.

77 Felding-Habermann B, O'Toole TE, Smith JW et al. Integrin activation controls metastasis in human breast cancer. *Proc Natl Acad Sci USA* 2001; 98: 1853–1858.

78 Bevilacqua MP, Nelson RM. Selectins. *J Clin Invest* 1993; 91: 379–387.

79 Kansas GS. Selectins and their ligands: current concepts and controversies. *Blood* 1996; 88: 3259–3287.

80 Laszik Z, Jansen PJ, Cummings RD, Tedder TF, McEver RP, Moore KL. P-selectin glycoprotein ligand-1 is broadly expressed in cells of myeloid, lymphoid, and dendritic lineage and in some nonhematopoietic cells. *Blood* 1996; 88: 3010–3021.

81 Hoff SD, Matsushita Y, Ota DM et al. Increased expression of sialyl-dimeric LeX antigen in liver metastases of human colorectal carcinoma. *Cancer Res* 1989; 49: 6883–6888.

82 Matsushita Y, Cleary KR, Ota DM, Hoff SD, Irimura T. Sialyl-dimeric Lewis-X antigen expressed on mucin-like glycoproteins in colorectal cancer metastases. *Lab Invest* 1990; 63: 780–791.

83 Matsusako T, Muramatsu H, Shirahama T, Muramatsu T, Ohi Y. Expression of a carbohydrate signal, sialyl dimeric Le(x) antigen, is associated with metastatic potential of transitional cell carcinoma of the human urinary bladder. *Biochem Biophys Res Commun* 1991; 181: 1218–1222.

84 Nakamori S, Kameyama M, Imaoka S et al. Increased expression of sialyl Lewis × antigen correlates with poor survival in patients with colorectal carcinoma: clinicopathological and immunohistochemical study. *Cancer Res* 1993; 53: 3632–3637.

85 Nakayama T, Watanabe M, Katsumata T, Teramoto T, Kitajima M. Expression of sialyl Lewis(a) as a new prognostic factor for patients with advanced colorectal carcinoma. *Cancer* 1995; 75: 2051–2056.

86 Nakamori S, Furukawa H, Hiratsuka M et al. Expression of carbohydrate antigen sialyl Le(a): a new functional prognostic factor in gastric cancer. *J Clin Oncol* 1997; 15: 816–825.

87 Kannagi R. Carbohydrate-mediated cell adhesion involved in hematogenous metastasis of cancer. *Glycoconj J* 1997; 14: 577–584.

88 Izumi Y, Taniuchi Y, Tsuji T, Smith CW, Nakamori S, Fidler IJ,

Cambridge Books Online © Cambridge University Press, 2009

Irimura T. Characterization of human colon carcinoma variant cells selected for sialyl Lex carbohydrate antigen: liver colonization and adhesion to vascular endothelial cells. *Exp Cell Res* 1995; 216: 215–221.

89 Nakamori S, Kameyama M, Imaoka S et al. Involvement of carbohydrate antigen sialyl Lewis(x) in colorectal cancer metastasis. *Dis Colon Rectum* 1997; 40: 420–431.

90 Kojima N, Handa K, Newman W, Hakomori S. Inhibition of selectin-dependent tumor cell adhesion to endothelial cells and platelets by blocking O-glycosylation of these cells. *Biochem Biophys Res Commun* 1992; 182: 1288–1295.

91 Rice GE, Bevilacqua MP. An inducible endothelial cell surface glycoprotein mediates melanoma adhesion. *Science* 1989; 246: 1303–1306.

92 Kim YJ, Borsig L, Han HL, Varki NM, Varki A. Distinct selectin ligands on colon carcinoma mucins can mediate pathological interactions among platelets, leukocytes, and endothelium. *Am J Pathol* 1999; 155: 461–472.

93 Mannori G, Crottet P, Cecconi O et al. Differential colon cancer cell adhesion to E-, P-, and L-selectin: role of mucin-type glycoproteins. *Cancer Res* 1995; 55: 4425–4431.

94 Goetz DJ, Ding H, Atkinson WJ et al. A human colon carcinoma cell line exhibits adhesive interactions with P-selectin under fluid flow via a PSGL-1-independent mechanism. *Am J Pathol* 1996; 149: 1661–1673.

95 Stone JP, Wagner DD. P-selectin mediates adhesion of platelets to neuroblastoma and small cell lung cancer. *J Clin Invest* 1993; 92: 804–813.

96 Aigner S, Sthoeger ZM, Fogel M et al. CD24, a mucin-type glycoprotein, is a ligand for P-selectin on human tumor cells. *Blood* 1997; 89: 3385–3395.

97 Kim YJ, Borsig L, Varki NM, Varki A. P-selectin deficiency attenuates tumor growth and metastasis. *Proc Natl Acad Sci USA* 1998; 95: 9325–9330.

98 Friederichs J, Zeller Y, Hafezi-Moghadam A, Grone HJ, Ley K, Altevogt P. The CD24/P-selectin binding pathway initiates lung arrest of human A125 adenocarcinoma cells. *Cancer Res* 2000; 60: 6714–6722.

99 McCarty OJ, Mousa SA, Bray PF, Konstantopoulos K. Immobilized platelets support human colon carcinoma cell tethering, rolling, and firm adhesion under dynamic flow conditions. *Blood* 2000; 96: 1789–1797.

100 Folkman J. Antiangiogenic therapy. In DeVita VT, Hellman S, Rosenberg SA, eds. *Cancer: principles and practice of oncology*, 5th edn. Philadelphia: Lippincott-Raven Publishers. 1997; 3075–3085.

101 Verheul HM, Hoekman K, Luykx-de Bakker S et al. Platelet: transporter of vascular endothelial growth factor. *Clin Cancer Res* 1997; 3: 2187–2190.

102 Pinedo HM, Verheul HM, D'Amato RJ, Folkman J. Involvement of platelets in tumour angiogenesis? *Lancet* 1998; 352: 1775–1777.

103 English D, Welch Z, Kovala AT et al. Sphingosine 1-phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and provides a novel link between hemostasis and angiogenesis. *FASEB J* 2000; 14: 2255–2265.

104 Nieswandt B, Hafner M, Echtenacher B, Mannel DN. Lysis of tumor cells by natural killer cells in mice is impeded by platelets. *Cancer Res* 1999; 59: 1295–1300.

105 Borsig L, Wong R, Feramisco J, Nadeau DR, Varki NM, Varki A. Heparin and cancer revisited: Mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. *Proc Natl Acad Sci USA* 2001; 98: 3352–3357.

106 Pearlstein E, Ambrogio C, Karpatkin S. Effect of antiplatelet antibody on the development of pulmonary metastases following injection of CT26 colon adenocarcinoma, Lewis lung carcinoma, and B16 amelanotic melanoma tumor cells into mice. *Cancer Res* 1984; 44: 3884–3887.

107 Ordinas A, Diaz-Ricart M, Almirall L, Bastida E. The role of platelets in cancer metastasis. *Blood Coagul Fibrinolysis* 1990; 1: 707–711.

108 Wood S Jr, Hilgard P. Aspirin and tumour metastasis. *Lancet* 1972; ii: 1416–1417.

109 Honn KV, Cicone B, Skoff A. Prostacyclin: a potent antimetastatic agent. *Science* 1981; 212: 1270–1272.

110 Karpatkin S, Ambrogio C, Pearlstein E. Lack of effect of in vivo prostacyclin on the development of pulmonary metastases in mice following intravenous injection of CT26 colon carcinoma, Lewis lung carcinoma, or B16 amelanotic melanoma cells. *Cancer Res* 1984; 44: 3880–3883.

111 Costantini V, Fuschiotti P, Allegrucci M, Agnelli G, Nenci GG, Fioretti MC. Platelet–tumor cell interaction: effect of prostacyclin and a synthetic analog on metastasis formation. *Cancer Chemother Pharmacol* 1988; 22: 289–293.

112 Honn KV, Onoda JM, Menter DG et al. Possible strategies for antimetastastic therapy. *Progr Clin Biol Res* 1986; 212: 217–249.

113 Vicenzi E, Lampugnani MG, Bolognese Dalessandro AP, Niewiarowska A, de Gaetano G, Donati MB. Dissociation between thromboxane generation and metastatic potential in cells from a murine fibrosarcoma. Studies with a selective thromboxane synthase inhibitor. *Int J Cancer* 1987; 39: 488–491.

114 Honn KV, Onoda JM, Pampalona K et al. Inhibition by dihydropyridine class calcium channel blockers of tumor cell–platelet–endothelial cell interactions in vitro and metastasis in vivo. *Biochem Pharmacol* 1985; 34: 235–241.

115 Lichtner RB, Hutchinson G, Wedderburn N, Hellmann K. Antiplatelet pyrimido-pyrimidines and metastasis. *Cancer Treat Rev* 1985; 12: 221–234.

116 Philips MR, Buyon JP, Winchester R, Weissmann G, Abramson SB. Up-regulation of the receptor (CR3) is neither necessary nor sufficient to promote neutrophil aggregation. *J Clin Invest* 1988; 82: 495–501.

117 Knudsen KA, Smith L, Smith S, Karczewski J, Tuszynski GP. Role of IIb–IIIa–like glycoproteins in cell–substratum adhesion of human melanoma cells. *J Cell Physiol* 1988; 136: 471–478.

118 Ruoslahti E, Giancotti FG. Integrins and tumor cell dissemination. *Cancer Cells* 1989; 1: 119–126.

Cambridge Books Online © Cambridge University Press, 2009

119 Humphries MJ, Olden K, Yamada KM. A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. *Science* 1986; 233: 467–470.
120 Humphries MJ, Yamada KM, Olden K. Investigation of the biological effects of anti-cell adhesive synthetic peptides that inhibit experimental metastasis of B16–F10 murine melanoma cells. *J Clin Invest* 1988; 81: 782–790.
121 Ugen KE, Mahalingam M, Klein PA, Kao KJ. Inhibition of tumor cell-induced platelet aggregation and experimental tumor metastasis by the synthetic Gly–Arg–Gly–Asp–Ser peptide. *J Natl Cancer Inst* 1988; 80: 1461–1466.
122 Bretti S, Neri P, Lozzi L, Rustici M, Comoglio P, Giancotti F, Tarone G. Inhibition of experimental metastasis of murine fibrosarcoma cells by oligopeptide analogues to the fibronectin cell-binding site. *Int J Cancer* 1989; 43: 102–106.
123 Saiki I, Murata J, Iida J, Nishi N, Sugimura K, Azuma I. The inhibition of murine lung metastasis by synthetic polypeptides [poly(arg–gly–asp) and poly(tyr–ile–gly–ser–arg)] with a core sequence of cell adhesion molecules. *Br J Cancer* 1989; 59: 194–197.
124 Charon MH, Poggi A, Donati MB, Marguerie G. Synthetic peptide with antithrombotic activity. In Rivier JE, Marshall GR, eds. *Peptides, chemistry, structure and biology*. Leiden: Escom. 1990: 82–83.
125 Poggi A, Vicenzi E, Cioce V, Wasteson A. Platelet contribution to cancer cell growth and migration: the role of platelet growth factors. *Haemostasis* 1988; 18: 18–28.
126 Dennis MS, Hezel WJ, Pitti RM et al. Platelet glycoprotein IIb–IIIa protein antagonists from snake venoms: evidence for a family of platelet-aggregation inhibitors. *Proc Natl Acad Sci USA* 1989; 87: 2471–2475.
127 Gould RJ, Polokoff MA, Friedman PA, Huang TF, Holt JC, Cook JJ, Niewiarowski S. Disintegrins: a family of integrin inhibitory proteins from viper venoms. *Proc Soc Exp Biol Med* 1990; 195: 168–171.
128 Knudsen KA, Tuszynski GP, Huang TF, Niewiarowski S. Trigramin, an RGD-containing peptide from snake venom, inhibits cell–substratum adhesion of human melanoma cells. *Exp Cell Res* 1988; 179: 42–49.
129 Rucinski B, Niewiarowski S, Holt JC, Soszka T, Knudsen KA. Batroxostatin, an Arg–Gly–Asp-containing peptide from *Bothrops atrox*, is a potent inhibitor of platelet aggregation and cell interaction with fibronectin. *Biochim Biophys Acta* 1990; 1054: 257–262.
130 Sheu JR, Lin CH, Chung JL, Teng CM, Huang TF. Triflavin, an Arg–Gly–Asp-containing antiplatelet peptide inhibits cell–substratum adhesion and melanoma cell-induced lung colonization. *Jpn J Cancer Res* 1992; 83: 885–893.
131 Beviglia L, Poggi A, Rossi C et al. Mouse antithrombotic assay. Inhibition of platelet thromboembolism by disintegrins. *Thromb Res* 1993; 71: 301–315.
132 Soszka T, Knudsen KA, Beviglia L, Rossi C, Poggi A, Niewiarowski S. Inhibition of murine melanoma cell–matrix adhesion and experimental metastasis by albolabrin, an RGD-containing peptide isolated from the venom of *Trimeresurus albolabris*. *Exp Cell Res* 1991; 196: 6–12.
133 Beviglia L, Stewart GJ, Newiarowski S. Use of a disintegrins to correlate cell adhesion and lung metastases in murine model. *J Cell Biochem* 1992; Suppl 16F: 165.
134 Scarborough RM, Naughton MA, Teng W et al. Design of potent and specific integrin antagonists. Peptide antagonists with high specificity for glycoprotein IIb–IIIa. *J Biol Chem* 1993; 268: 1066–1073.

Cambridge Books Online © Cambridge University Press, 2009

55

Platelets and renal diseases

Paola Boccardo[1], Miriam Galbusera[1] and Giuseppe Remuzzi[2]

[1]*Mario Negri Institute for Pharmacological Research, Via Gavazzeni 11, 24125 Bergamo, Italy*

[2]*Unit of Nephrology and Dialysis Azienda Ospedaliera, Ospedali Riuniti di Bergamo, Largo Barozzi 1, 24128 Bergamo, Italy*

Platelets in uremia

Morgagni in 1764 in his *Opera Omnia*[1] was the first to recognize the remarkable association between bleeding and renal dysfunction. Bleeding may be a serious complication of acute and chronic renal failure[2,3], and since the first review in 1907 on the association between uremia and abnormal bleeding, the clinical manifestations of uremic bleeding have been well described[4–7]. Hemorrhagic complications varying from ecchymoses, epistaxis, bleeding from gums and venipuncture sites, and overt gastrointestinal bleeding have been observed in up to one-third of uremic patients[5,8,9], however, low-grade gastrointestinal bleeding may be even more common[10].

Bleeding became a clinical problem at the beginning of the dialysis era, when patients sometimes died from excessive bleeding from the gastrointestinal tract or abdominal organs[8,11].

With the advent of modern dialysis techniques and the use of erythropoietin to correct anemia[12] the frequency of severe hemorrhage has decreased; however, this complication still limits surgery and invasive procedures in these patients.

The cause of uremic bleeding has been the subject of a major debate since the 1970s. The pathogenesis is considered multifactorial (Table 55.1); however, platelet–platelet and platelet–vessel wall interactions appear to be of crucial importance. Abnormalities of blood coagulation and fibrinolysis partially corrected by dialysis predispose the uremic patients to thrombosis rather than bleeding. Chronic renal failure also seems to be associated with an increased incidence of arterial and venous thromboembolic complications and this chapter will review the platelet and vascular defects in uremia.

Table 55.1. Factors involved in the pathogenesis of bleeding associated with chronic renal failure

Platelet abnormalities
Subnormal dense granule content
Reduction in intracellular ADP and serotonin
Impaired release of the platelet α-granule protein and β-thromboglobulin
Enhanced intracellular c-AMP
Abnormal mobilization of platelet Ca^{2+}
Abnormal platelet arachidonic acid metabolism
Abnormal ex vivo platelet aggregation in response to different stimuli
Defective cyclooxygenase activity
Abnormality of the activation-dependent binding activity of GP IIb–IIIa
Uremic toxins, especially parathyroid hormone
Abnormal platelet–vessel wall interactions
Abnormal platelet adhesion
Increased formation of vascular PGI_2
Altered von Willebrand factor
Anemia
Altered blood rheology
Erythropoietin deficiency
Abnormal production of nitric oxide
Drug treatment
β-Lactam antibiotics
Third-generation cephalosporins
Non-steroidal anti-inflammatory drugs

Thrombocytopenia

Thrombocytopenia is found in 16–55% of uremic patients[6,8,9,13–19], suggesting platelet overconsumption or inadequate production[20]. However, platelet number is rarely less than 80 000/μl[5,6,8,9,14,21–23], a concentration generally considered adequate for normal hemostasis.

The interaction of blood with biocompatible (e.g. cuprophane) hemodialysis membranes leads to complement activation, transient neutropenia, and significant thrombocytopenia during dialysis[24,25]. This phenomenon does not occur when biocompatible membranes, which do not activate complement, are used[24,25]. Heparin therapy, used to inhibit clotting in the extracorporeal circuit, can activate platelets and can occasionally induce thrombocytopenia by an immunologic mechanism[26,27].

A major decrease in platelet count is present in patients with renal failure associated with lupus vasculitis, hemolytic uremic syndrome/thrombotic thrombocytopenic purpura, disseminated intravascular coagulation, eclampsia, and renal allograft rejection.

The mean platelet volume may also be decreased in uremia. This decrease in the circulating platelet mass is inversely related to the bleeding time in uremic individuals[28].

Platelet abnormalities

Dense granule content is subnormal in uremic platelets[29,30]; the storage pool defect is denoted by the reduction in platelet ADP and serotonin and by the increase in the ratio of ATP to ADP. Cyclic AMP (cAMP) is elevated in patients with terminal renal insufficiency and hemodialysis partially corrects this abnormality[31]. Since ADP and serotonin are platelet agonists and cyclic AMP is an inhibitor of platelet function, this imbalance could contribute to an activation defect. The release of ATP in response to thrombin is also reduced in uremia[29] suggesting a defective secretory mechanism. Calcium content is increased in uremic platelets[32], which also mobilize calcium abnormally in response to stimulation[33]. Elevation in platelet cAMP and abnormal calcium mobilisation drew attention to the possibility that parathyroid hormone (PTH) may play a role in uremic platelet dysfunction. This speculation was supported by the observation that PTH inhibits platelet aggregation in vitro[34,35]. However, the bleeding time does not correlate with serum concentrations of intact PTH or PTH fragments[36], suggesting that elevated PTH in renal failure patients is not likely to play a major role in the uremic platelet defect. An impairment in the release of the platelet α-granule proteins and β-thromboglobulin was demonstrated in hemodialysis patients[37].

Defective platelet aggregation in response to various stimuli such as ADP, epinephrine, collagen, and thrombin is documented in a great number of studies[11,20,22,29,38–44], although the degree of impairment of platelet aggregation in uremia varies considerably, and in several reports platelet aggregation was found to be normal or increased[45–48].

Impaired synthesis by uremic platelets of thromboxane A_2 (TxA_2), a potent enhancer of both platelet release and aggregation, has been documented, but the nature of this abnormality remains controversial[29,30,49–54]. A decreased platelet aggregation and TxA_2 formation in response to ADP, collagen, and thrombin as well as to arachidonic acid, consistent with a defect in platelet prostaglandin synthesis at or beyond the cyclooxygenase level was observed by some investigators[51,52]. Others found normal TxA_2 formation by uremic platelets in response to arachidonic acid as well as to the TxA_2 analogue, U-46619, while TxA_2 synthesis in response to ADP, collagen, or the combination of collagen with ADP or epinephrine was impaired[29,50,53]. These findings would be consistent with a defect in the stimulated release of arachidonic acid from platelets. The observation that these defects are corrected by dialysis, indicates that they are probably due to a factor present in uremic plasma[29,52,53].

Platelet–vessel wall interaction

Normal platelet adhesion requires initial platelet contact followed by platelet spreading on the subendothelial matrix. Two adhesive proteins, fibrinogen and von Willebrand factor (vWF), and two platelet adhesion receptors, glycoprotein (GP) Ib and the $\alpha_{IIb}\beta_3$ complex play a vital role in the formation of platelet thrombi at sites of injury. At high shear rates, such as those found in the capillary circulation, contact is dependent on the binding of vWF to the platelet GP Ib[55,56]. This interaction is also necessary for ristocetin-induced platelet aggregation in vitro. Binding of GP Ib to vWF is normal in uremic patients[57], as is the surface expression of the receptor[57,58]. In patients with chronic renal failure, a decrease in the total content of platelet GP Ib has been documented[58,59], accompanied by an increase in soluble glycocalicin (a soluble proteolytic fragment of GP Ib), and is probably due to proteolytic damage to membrane GP Ib. The normal surface expression of this receptor and the total decrease in content account for a redistribution from the intraplatelet pool to the surface pool[57,58]. The activation-dependent receptor function of the $\alpha_{IIb}\beta_3$ complex is defective in uremia, as

Cambridge Books Online © Cambridge University Press, 2009

shown by decreased binding of both vWF and fibrinogen to stimulated platelets[57]. The number of $\alpha_{IIb}\beta_3$ receptors expressed on the platelet membrane is normal. Removal of substances present in uremic plasma markedly improved the $\alpha_{IIb}\beta_3$ defect. Thus, a reversible abnormality of the activation dependent binding activity of $\alpha_{IIb}\beta_3$ caused by a dialysable toxic substance or substances[57,60], or due to receptor occupancy by fibrinogen fragments present in uremic plasma[61], is probably a major component of the altered platelet function in uremia. The impaired $\alpha_{IIb}\beta_3$ activation in uremia may explain aggregation defects as well as reduced vWF-dependent adhesion and thrombus formation[62–64].

vWF levels in plasma, measured either by immunologic methods or functionally by ristocetin cofactor activity, are normal or elevated in renal failure[65], and qualitative abnormalities of vWF have not been uniformly observed[43,59,63,66].

The observation that cryoprecipitate[67] – a plasma derivative rich in vWF – and desmopressin[68] – a synthetic derivative of antidiuretic hormone that releases autologous vWF from storage sites – significantly shorten the bleeding time of uremic patients suggests that a functional defect in the vWF–platelet interaction may indeed play a role in the abnormal hemostasis of these patients.

Molecules such as PGI_2 and nitric oxide (NO) may affect platelet–vessel wall interaction thus amplifying the platelet defects observed in uremia.

An increased synthesis by vascular endothelium formation of prostacyclin (PGI_2), a potent vasodilator and inhibitor of platelet function, was documented in uremic patients[37,69] and blood vessels from rats with experimentally induced uremia[70,71]. Furthermore, plasma from uremic patients stimulates PGI_2 generation by cultured endothelial cells more than normal plasma[72].

NO is a potent modulator of vascular tone that limits platelet adhesion to endothelium[73] and platelet–platelet interaction increasing the formation of cell cyclic GMP[74–76]. Data are available showing that platelets from uremic patients on hemodialysis generate more NO than healthy subjects[77,78]. Uremics have higher levels of NO in exhaled air[79] and higher plasma levels of NO metabolites[80–83] than normal humans. In addition, plasma from chronic hemodialysed patients potently induces NO synthesis from human umbilical vein endothelial cells (HUVEC)[78] and cultured human microvascular endothelial cells[84]. The stimulatory activity found in uremic plasma is attributed to cytokines such as TNFα and IL-1β that are potent inducers of the inducible isoform of NO synthase and circulate in increased amounts in the plasma of patients with chronic renal failure who either are, or are not, on maintenance hemodialysis[77,85–88].

Dialysis

Several dialysable uremic toxins, e.g. urea, creatinine, phenol, phenolic acids, or guanidinosuccinic acid (GSA) may possibly be involved in the genesis of the uremic platelet dysfunction[89–91]. GSA, which accumulates in uremic plasma, inhibits the second wave of platelet aggregation to ADP when added to normal platelet-rich plasma[90], and as recently demonstrated GSA is involved in the generation of NO[78]. The effect of GSA of stimulating NO release provides a biological explanation of the old data of early 1970 showing that, among uremic toxins, GSA was the only one that consistently inhibited platelet function to such a degree that it was defined the 'x' factor in uremic bleeding[92,93].

Phenolic acid, at the concentrations found in uremic plasma, also impairs primary aggregation to ADP[89].

All these observations suggest that reducing the blood levels of these compounds may correct the abnormal hemostasis of patients with renal failure. However, no correlation has been found between bleeding time or platelet adhesion and serum levels of the dialysable metabolites mainly accumulating in uremia[91].

Dialysis improves platelet functional abnormalities and reduces, but does not eliminate the risk of hemorrhage[49]. Hemodialysis can even contribute to the bleeding. The continuous platelet activation induced by the interaction between blood and artificial surfaces, and the release of platelet-derived proteins, can induce platelet 'exhaustion' and consequently favour bleeding in uremic patients. Peritoneal dialysis has been suggested to be more effective than hemodialysis in removing uremic toxins that adversely affect platelet function, however this procedure may cause platelet hyper-reactivity, which in some cases may be related to hypoalbuminemia[94].

Role of anemia

Platelet adhesion and aggregation in flowing systems[56,95] are markedly potentiated by red blood cells. Erythrocytes enhance platelet function by releasing ADP[96], by inactivating PGI_2[97], and by increasing platelet–vessel wall contact by displacing platelets away from the axial flow and toward the vessel wall[95]. The independent role of anemia in the bleeding tendency of uremia has been extensively investigated. A significant negative correlation was found between bleeding time and packed cell volume (PCV)[98]. In fact, despite a shorter bleeding time, a significant negative correlation between hematocrit and bleeding time was also demonstrable in 15 non-uremic anemic patients.

Cambridge Books Online © Cambridge University Press, 2009

These results were subsequently confirmed by other studies[99,100] that found that anemia was the main determinant of the prolonged bleeding time in uremic patients.

The cloning of the human erythropoietin gene and the production of the human recombinant erythropoietin (rhEPO) have provided clinicians with a powerful tool to correct the anemia associated with the renal failure. Clinical trials have provided evidence that rhEPO reverses the anemia of renal failure and eliminates any dependency upon transfusion[101]. Increasing doses of rhEPO, when given to hemodialysis patients with a history of bleeding, severe anemia (hematocrit less than 23%), and the long basal bleeding time (more than 19 minutes), induced a progressive increase in hematocrit accompanied by significant shortening of the bleeding time[12]. Although improvement in platelet adhesion to subendothelium was observed in some studies, no consistent changes in platelet number, platelet aggregability, markers of platelet activation in plasma, platelet TxA_2 formation, platelet adenine nucleotide content, global coagulation test results, antithrombin III, or cross-linked fibrin derivatives were reported[12,102,103].

The efficacy of erythropoietin is indisputable with improvement in the quality of life, cardiac function, physical work capacity, cognitive function and sexual function with hematocrit of 36% to 39%[104]; however the benefits and risks of complete correction of anemia (hematocrit 38% to 42%) and the optimal target concentration have not yet been established. Recently, the safety of the long-term maintenance of a normal hematocrit has been questioned, as a consequence, in part, of the early termination of the Normal Hematocrit Cardiac Trial[105], in which the patients randomly assigned to the normal hematocrit group presented with a higher mortality and a higher incidence of non-fatal myocardial infarction[106].

Effect of drugs

Uremic patients may be at an increased risk of bleeding complications caused by drug treatment. The risk of bleeding associated with the accumulation of β-lactam antibiotics in uremia has been highlighted[107]. β-lactam antibiotics apparently act by perturbing platelet membrane function and by interfering with ADP receptors[108,109]. The prolonged bleeding time and the abnormal platelet aggregation are related to the dose and duration of treatment and are promptly reversible after discontinuation. Third-generation cephalosporins may also inhibit platelet function and may lead to marked disturbances of blood coagulation[110,111].

Risk of bleeding in uremic patients is also associated with aspirin given to prevent vascular access thrombosis[112] or platelet activation on dialysis membranes[113]. The beneficial effect of aspirin on vascular access thrombosis can be achieved with a moderate dose of 160 mg/d, which inhibits platelet TxA_2 generation without affecting vascular PGI_2 formation[112]. However, a moderate dose of aspirin may prolong the bleeding time to a greater extent in uremic than in control subjects[114,115]. This difference appears not to be related to increased susceptibility of cyclooxygenase in uremic platelets. Furthermore, a temporal dissociation was found in uremic patients between the prolongation of bleeding time and inhibition of serum TxA_2 generation after aspirin. Indeed aspirin seems to have two distinct inhibitory effects on platelet function in uremia: a transient effect which interferes with one of the determinants of bleeding time, and the lasting effect due to the irreversible blocking of platelet cyclooxygenase[115]. However, the prolongation of bleeding caused by aspirin may explain the frequency of gastrointestinal bleeding in uremic patients[116,117]. Thus the use of aspirin for uremic patients treated with rhEPO to prevent thrombotic complications associated with an increased hematocrit is highly questionable.

Nonsteroidal anti-inflammatory drugs such as indomethacin, ibuprofen, naproxen, phenylbutazone, and sulfinpyrazone also inhibit platelet cyclooxygenase[118] and disturb platelet function[119]. However, in contrast to aspirin, the inhibitory effect of these compounds on platelet cyclooxygenase is readily reversible as the blood concentration of the drugs falls upon cessation of administration[119].

Therapeutic strategies to correct uremic bleeding

Guidelines for the management of hemorrhagic complications of uremia are summarized in Table 55.2.

Thrombotic complications

Paradoxically, hemodialysis exposes uremic patients to the thrombotic complications of vascular access. Percutaneous cannulation, arteriovenous shunt, and native vein or prosthetic arteriovenous fistula, and fistulas made of artificial polymers used for chronic hemodialysis are particularly prone to thrombotic occlusion, which accounts for a substantial percentage of hospital admission of dialysis patients[120]. It has been estimated that more than half of all grafts develop thrombosis within 2 years[121]. To prevent

Cambridge Books Online © Cambridge University Press, 2009

Table 55.2. Guidelines for the management of hemorrhagic complications of uremia

For all patients with hemorrhagic complications or undergoing major surgery, the adequacy of dialysis should be appropriately checked. It is also advisable to change the dialysis schedule for 1 to 2 mo in patients who have experienced severe hemorrhages (such as major gastrointestinal bleeding, hemorrhagic pericarditis, subdural hematomas) or who have undergone recent cardiovascular surgery, so that heparin can be avoided.
Acute bleeding episodes may be treated with desmopressin at a dose of 0.3 μg/kg intravenously (added to 50 ml of saline over 30 min) or subcutaneously. Intranasal administration of this drug at a dose of 3 mg/kg is also effective and is well tolerated. The effect of desmopressin lasts only a few hours, a major limitation to its use in treating severe hemorrhage, and desmopressin appears to lose efficacy when repeatedly administered. Because the favourable effect of cryoprecipitate on bleeding time has not been uniformely observed, we do not recommend its use.
The ideal treatment of persistent chronic bleeding should have a long-lasting effect. Conjugated estrogen treatment given by intravenous infusion in a cumulative dose of 3 μg/kg as daily divided dose (i.e. 0.6 mg/kg for 5 consecutive days) is the most appropriate way of achieving long-lasting hemostatic competence.
Severely anemic patients should receive blood or red blood cell transfusions to improve hematocrit values. Red blood cell transfusion is hemostatically effective only when the hematocrit rises above 30%. As an alternative, bleeding in patients with renal failure and hematocrit less than 30% can be treated successfully with erythropoietin, according to the following indications. Before therapy, iron stores should be assessed by determination of serum ferritin, serum iron, and total iron-binding capacity (TIBC). Patients with microcytic anemia and normal iron stores should be evaluated for aluminium toxicity and thalassemia. Uncontrolled hypertension is a contraindication to the initiation of rhEPO therapy. Patients must be urged to adhere to pre-hEPO dietary restriction. The hemoglobin level or hematocrit should be measured each week during induction of therapy and every 2 wk thereafter. Serum iron, TIBC and serum ferritin should be measured monthly for 3 mo and every 2 to 3 mo thereafter.

vascular access thrombosis, antiplatelet agents such as low-dose aspirin[112,113], sulfinpyrazone[122,123] or ticlopidine[124–126] have been used, with encouraging results, but concerns about the possibility of increases in bleeding complications have limited the routine use of these agents in dialysis centres.

Cardiovascular events related to thrombosis are a predominant cause of death and account also for an important morbidity in uremic patients both on conservative treatments, or on replacement of renal function by dialysis or transplantation. It is evident that the vast majority of patients with chronic uremia has several risks for developing cardiovascular disease, either pre-existing or caused or aggravated by chronic renal failure, such as hyperlipidemia, hypertension, glucose intolerance and hyperparathyroidism. In addition, hemodialysis itself may contribute to lipid abnormalities that are present in every type of chronic renal disease[120,127]. Hemostatic abnormalities consistent with a hypercoagulable state have been widely described in patients with end-stage renal failure on hemodialysis. Risk factors include enhanced platelet aggregability, increased plasma fibrinogen, Factor VIII: C and vWF, decreased protein C anticoagulant activity and protein S, impaired fibrinolytic system activity, raised plasma lipoprotein(a), increased plasmatic concentration of homocysteine, and the presence of lupus anticoagulant (for review see Joist et al.[128]).

The association between renal vein thrombosis (RVT) and the nephrotic syndrome (NS) has been long recognized[129,130]. The incidence of RVT appears to be substantially higher in NS caused by membranous glomerulonephritis than with membranoproliferative glomerulonephritis or other forms of renal disease[129–133]. A variety of hemostatic alterations, consistent with a hypercoagulable state, have been reported in NS[130,134]. Increased platelet aggregability to ADP and collagen was found in nephrotic children[135]. Platelet defects[128] vary from spontaneous platelet aggregation, enhanced response to arachidonic acid, increased production and release of arachidonic acid, and shortened platelet survival. Platelet hyperaggregability in NS has generally been attributed to hypoalbuminemia because albumin inhibits arachidonic acid-induced platelet aggregation and arachidonic acid conversion from platelet phospholipids to endoperoxide intermediates and TxA_2. However, no apparent relationship between hyperaggregability and hypoalbuminemia has been found in some studies[129,136–139]. Hypocalcemia and hypercholesterolemia may be involved as well in platelet hyperaggregability in NS[127,137,140–142]. The complex interaction between the effects on platelet function in uremia, the underlying glomerulonephritis, hemodialysis, and medications may account for the lack of a statistically significant relationship between platelet hyperaggregability, hypoalbuminemia, and/or hypercholesterolemia in patients with NS. In these patients,

Cambridge Books Online © Cambridge University Press, 2009

Table 55.3. Platelet secretory products involved in the pathogenesis of glomerular disease

α-granule constituents
Platelet-derived growth factor
Platelet factor 4
Transforming growth factor β
Basic fibroblast growth factor
Epidermal growth factor
β-thromboglobulin
Hepatocyte growth factor
Fibronectin
Thrombospondin
Lysosomial constituents
Collagenase
Cathepsins
Elastase
Heparitinase

platelet abnormalities in association with the common modest thrombocytosis, with increased levels of coagulation factors, and with reduced levels of antithrombin III may play an important role in the development of RVT and other venous and arterial thromboembolic complications[128].

Platelets in glomerular diseases

Platelets are involved in the pathogenesis of glomerular diseases through a variety of mechanisms[143,144]. The release of platelet activating factor (PAF) and other platelet secretory products (Table 55.3) may alter glomerular permeability to proteins and enhance immune-mediated glomerular injury. In addition platelet-derived growth factors may account for the renal disease progression via their chemotactic activity for infiltrating leukocytes and their effect of promoting extracellular matrix synthesis by resident renal cells. Evidence of platelet activation in renal diseases has been demonstrated by the clinical observation of shortened platelet survival or intrarenal platelet sequestration in patients with membranoproliferative, membranous or lupus glomerulonephritis, focal segmental glomerulosclerosis and diabetic nephropathy[145–147]. In diffuse proliferative glomerulonephritis accelerated platelet destruction has been found to be prominent and correlations between platelet destruction and intraglomerular cellular proliferation were reported[146–148]. In patients with lupus nephritis thrombocytopenia and glomerular thrombosis, factors important in determining the subsequent development of glomerulosclerosis, are common findings[148,149]. Platelet and fibrin thrombi in lumens of small arteries and glomerular capillaries are also common renal pathologic features in hemolytic uremic syndrome/thrombotic thrombocytopenic purpura, systemic sclerosis, acute and chronic transplant rejection, and nephropathy associated with essential hypertension[150–153]. Intraglomerular platelet aggregates were found also in patients with mesangiocapillary glomerulonephritis and IgA nephropathy[154–158].

Platelet secretory products as a cause of glomerular permeability alteration and glomerular injury amplification

There is evidence that during glomerulonephritis platelets are activated and their secretory products localise in glomerular structures, however little is known about the effects of these platelet constituents and nearly all of our understanding derives from animal experiments.

The initiation of immune vascular injury involves a complex array of inflammatory mediators, including PAF, which may play a central role. PAF is a potent phospholipid autacoid, initially isolated from stimulated basophils, that is synthesized by different cell types, including neutrophils, monocytes, endothelial cells and glomerular mesangial cells[159,160]. PAF is also secreted by platelets and, in turn, triggers platelet aggregation, activation and degranulation[161]. Biological effects of PAF that may determine glomerular injury include chemotaxis and activation of leukocytes, complement activation, contraction and stimulation of mesangial cells to produce eicosanoids and radical oxygen species. Evidence that PAF is involved in glomerular injury derives from studies in different experimental models of glomerulonephritis: increased glomerular production of PAF and improvement of the glomerular disease by a specific PAF receptor antagonist was observed in nephrotoxic nephritis (NTN)[162–164], acute serum sickness[165], anti-Thy-1 glomerulonephritis[166] and murine lupus nephritis[167,168]. Increased plasma and urinary levels of PAF correlated with proteinuria and renal histological abnormalities in murine lupus nephritis[169] and, in this model, reduced proteinuria and improved survival were observed after the chronic administration of a PAF receptor antagonist[168].

Other platelet secretory products that are implicated in proteinuria and immune complex localization by virtue of their ability to alter glomerular permeability are endogenous polycationic macromolecules, platelet factor 4 (PF4) and β-thromboglobulin. They may bind to glomerular polyanions, neutralizing the electrostatic barrier to circulating macromolecules[170,171]. In experimental animal models such as rabbits with acute serum sickness and

Cambridge Books Online © Cambridge University Press, 2009

after infusion of bovine serum albumin in pre-immunized animals, the loss of glomerular polyanions together with glomerular localization of platelet cationic proteins and immune complexes has been documented[172,173]. Loss of polyanions has also been reported in several human glomerular diseases[171,174,175]. The causes of the loss of glomerular polyanions in glomerulonephritis have not been fully elucidated. However platelet products could interfere with glomerular polyanions by different processes, including neutralization of fixed negative charges by polycations, enzymatic degradation of polyanions and reduction in glomerular cell synthesis or quality of basement membrane constituents such as type IV collagen, fibronectin and heparan sulfate. The latter molecules are sensitive to the action of elastase, collagenase and heparatinase, which are released by platelets upon stimulation[176]. Infusion of collagenase, heparatinase, elastase or cathepsin G into the renal vasculature resulted in increased glomerular permeability to tracer molecules or development of proteinuria.

Platelet-derived growth factors as mediators of renal disease progression in experimental and human glomerulopathies

A variety of cytokines and growth factors that are secreted by platelets directly influence glomerular mesangial cell functions, including proliferation, migration, contraction and extracellular matrix protein synthesis[177].

Platelet-derived growth factor (PDGF) is a 30-kilodalton glycoprotein that appears to mediate mesangial cell proliferation in glomerulonephritis[177,178]. In response to activation by numerous substances, including PAF, thrombin, collagen and immune complexes[179], platelets release PDGF – stored in the α-granules – into the extracellular environment. PDGF is a potent mitogen for mesangial cells[180] and may have an additional role as an intermediary in the effects of other growth factors. Epidermal growth factor and basic fibroblast growth factor induce the PDGF gene in mesangial cells and exert their mitogenic effect at least partly through the PDGF loop[181]. Binding of PDGF to its receptor on mesangial cells activates phospholipase C with generation of inositol triphosphate, enhancement of intracellular calcium and diacylglycerol and protein kinase C activation[182]. PDGF together with other platelet secretory products such as fibronectin[183] and thrombospondin[184] act as chemoattractants for mesangial cells and this process appears important in conditions of mesangial cells interposition as in membranoproliferative glomerulonephritis. PDGF also regulates matrix production directly or by additional growth factor pathways, such as stimulating the release from mesangial cells of transforming growth factor beta (TGFβ) which, in turn, controls the synthesis of a variety of mesangial matrix proteins. Administration of an antibody to PDGF inhibited mesangial cell proliferation in the anti-Thy-1 model[185] suggesting that PDGF plays an important role in the progression of proliferative diseases. In rats with proliferative glomerulonephritis induced by anti-Thy-1 antibody[186], renal ablation[187] and streptozotocin-induced diabetes[188] it has been demonstrated that increased mesangial expression of PDGF and/or PDGF-β receptor as well as their respective mRNAs in glomeruli is a consequence of platelet activation.

Autocrine pathways involving peptide growth factors exist also in human glomerular disease. This evidence derives from findings that expression of PDGF and PDGFβ receptor is enhanced in vascular structures and glomeruli in diverse proliferative diseases such as IgA, crescentic glomerulonephritis, focal segmental glomerulosclerosis, chronic transplant rejection and lupus nephritis[189–192].

TGFβ is released from platelets locally at the site of injury and has a variety of biological actions[193–195], including induction of its own production by local cells. TGFβ is also a strong chemoattractant of inflammatory cells that generate TGFβ by themselves as part of the cytokine network. In its defensive role, TGFβ mediates the recruitment of leukocytes, stimulates extracellular matrix deposition promoting tissue repair and the resolution of inflammation. In this context TGFβ induces the production of fibronectin, collagens, proteoglycans and tenascin and, in the mean time, blocks matrix degradation by increasing the levels of protease inhibitors, such as plasminogen activator inhibitor-1, and by reducing the secretion of proteases[196]. On mesangial cell proliferation TGFβ displays a dual mode of action: it inhibits the proliferation of cells actively proliferating in low density, but is a potent mitogen for confluent cells[197,198]. Evidence that TGFβ has a role in inflammatory renal diseases rests on the fact that TGFβ is stored in high concentration in platelets[199] and is expressed in activated monocytes and macrophages infiltrating the glomerulus. In glomerulonephritis and glomerulosclerosis TGFβ is supposed to be the principal mediator of increased extracellular matrix synthesis[195,200]. A role for TGFβ in mediating tubulo-interstitial and glomerular damage in progressive nephropathies is supported by the following observations. Glomerular expression of TGFβ is increased together with matrix deposition in experimental and human diabetic nephropathy[201]. In the experimental adriamycin-induced chronic model of progressive renal disease, the development of glomerulosclerosis parallels the increase in mRNA TGFβ and TGFβ receptor expression and the

Cambridge Books Online © Cambridge University Press, 2009

enhanced secretion of TGFβ and is associated with raised mRNA levels of fibronectin in both cortex and glomeruli[202].

Thus, platelets appear to play a central role in the early events in glomerular disease, however perpetuation of the proliferation of resident cells in clinical disease may also involve other autocrine pathways.

Effect of antiplatelet agents on glomerular diseases

Depleting circulating platelets has been very effective in prevention of immune complex nephritis[162,176,203–205] and in inhibiting mesangial cell proliferation in several models of proliferative glomerular nephritis[206–208] and diabetic nephropathy[188]. In experimental and clinical glomerulonephritis the use of drugs against specific pathways of platelet activation and secretion ameliorates the progression of renal failure. These successful therapeutic results provide further evidence for the role of platelets as mediators of the progression of glomerular disease.

In 1972, treatment with cyclophosphamide, the anticoagulant warfarin and the platelet-inhibiting drug dipyridamole improved the prognosis in patients with membranoproliferative glomerulonephritis[209]. However, a multicentre prospective randomized Canadian study found no beneficial effect of this triple therapy[210].

The effects of drugs such as warfarin, dipyridamole, aspirin and their association derive from reports involving a small number of patients. A prospective trial of warfarin and dipyridamole was performed in 18 patients with membranoproliferative glomerulonephritis[211]. The unpaired analysis compared ten patients followed for an initial year with eight patients receiving treatment first. Renal function remained stable and proteinuria decreased in the treated group. Favourable effects of the treatment were observed only in 6 out of 13 patients who completed both the control and treatment year. Association with dipyridamole and aspirin was studied in 40 patients with type I membranoproliferative glomerulonephritis[212]. In treated patients glomerular filtration rate (GFR) was better maintained, platelet half-life was increased and progression to end-stage renal disease occurred less often and over a longer period with respect to the placebo group. The interpretation of these results is difficult because this finding could derive from reduction in platelet/vascular wall interaction or in platelet consumption or, alternatively, might have been related to inhibition of both the platelet and the renal production of TXA_2 and prostaglandins. However, even if inhibition of TXA_2 synthesis may have a beneficial effect, the simultaneous inhibition of vasodilatory prostaglandins should theoretically be deleterious to renal function.

Ticlopidine is a potent inhibitor of platelet function, with a broad spectrum of pharmacological actions on platelet and platelet/cell interactions[213,214]. This drug differs significantly from aspirin in its mechanism of antiplatelet action and in its toxicity profile. Ex vivo studies have shown that ticlopidine impairs fibrinogen binding to its platelet receptor $\alpha_{IIb}\beta_3$ complex, and inhibits platelet aggregation in response to many agonists, particularly ADP[213,214]. In vivo ticlopidine prolongs bleeding time in man and laboratory animals, causes red cell deformability and reduces blood viscosity. Ticlopidine has been used clinically to examine long-term drug effects in patients with chronic glomerular disease including focal glomerulosclerosis, membranous nephropathy and membranoproliferative and diffuse proliferative nephritis[215]. In such patients, proteinuria was reduced, platelet count improved and circulatory levels of β-thromboglobulin and PF4 decreased.

Although the findings in experimental animals provide a rationale for the use of ticlopidine in progressive glomerular diseases, the lack of controlled clinical trials makes it impossible to draw any conclusion about its efficacy. Moreover, caution in the use of this drug should be exercised because of the possibility of the induction of hemolytic uremic syndrome/thrombotic thrombocytopenic purpura, as a rare collateral effect[216–220].

The effect of certain foods, such as fish oil, on platelet function must be considered. Fish oil contains omega-3 fatty acids that act by reducing the platelet content of arachidonic acid and by competing with arachidonic acid for cyclooxygenase[221,222]. Since the first report of beneficial effects of fish oil therapy on IgA nephropathy[223] several controlled trials have been reported with rather inconsistent findings[223–228]. However, the largest prospective controlled study reported to date, from the Mayo Clinic, did demonstrate a positive effect of fish oil therapy[227]. By the end of treatment, 5% of treated patients versus 33% of the placebo group reached an increase of serum creatinine of at least 50%. There was no beneficial effect observed in urinary protein excretion. Because of its safety, fish oil therapy is an attractive approach for combination with other regimens.

A variety of therapies that inhibit platelet function have been shown to be effective in glomerular diseases despite the non-specific nature of their actions. Identification of specific platelet inhibitors or target interventions to specific platelet pathways, will be crucial for understanding the exact role of platelets and their products in glomerular diseases.

Cambridge Books Online © Cambridge University Press, 2009

Acknowledgement

The authors thank Ms Laura Arioli for assistance in preparing the manuscript.

References

1 Morgagni GB. Opera Omnia Ex *Typographia Remondiniana*. 1764; Venezia, Italy.
2 Rabiner SF. Uremic bleeding. *Prog Hemost Thromb* 1072; 1: 233–250.
3 Remuzzi G. Bleeding disorders in uremia: Pathophysiology and treatment. *Adv Nephrol Necker Hosp* 1989; 18: 171–186.
4 Riesman D. Hemorrhages in the course of Bright's disease with special reference to the occurrence of a hemorrhagic diathesis of nephritic origin. *Am J Med Sci* 1907; 134: 709.
5 Gross R, Nieth H, Mammen E. Blutungsbereitschaft und gerinnungstorungen bei uramie. *Klin Woschr* 1958; 136: 107–109.
6 Larsson SO. On coagulation and fibrinolysis in renal failure. *Scand J Haematol* 1971; 15 (Suppl.): 1–59.
7 Rabiner SF. Bleeding in uremia. *Med Clin North Am* 1972; 56: 221–233.
8 Lewis JH, Zucker MB, Ferguson JH. Bleeding tendency in uremia. *Blood* 1956; 11: 1073–1076.
9 Rath CE Mailliard JA, Schreiner GE. Bleeding tendency in uremia. *N Engl J Med* 1957; 257: 808–811.
10 Rosenblatt SG, Drake S, Fadem S, Welch R, Lifschitz MD. Gastrointestinal blood loss in patients with chronic renal failure. *Am J Kidney Dis* 1982; 1: 232–236.
11 Steiner RW, Coggins C, Carvalho AC. Bleeding time in uremia: a useful test to assess clinical bleeding. *Am J Hematol* 1979: 7: 107–117.
12 Moia M, Mannucci PM, Vizzotto L, Casati S, Cattaneo M, Ponticelli C. Improvement in the haemostatic defect of uraemia after treatment with recombinant human erythropoietin. *Lancet* 1987; ii: 1227–1229.
13 Stewart JH. Platelet numbers and life span in acute and chronic renal failure. *Thrombos Diath Haemorrh* 1967; 17: 532–542.
14 Cheney K, Bonnin JA. Haemorrhage, platelet dysfunction and other coagulation defects in uremia. *Br J Haematol* 1962; 8: 215–222.
15 Kulhback B. Bleeding tendency in chronic renal failure. *Acta Med Scand* 1957; 157: 173–177.
16 Bayer WL, Szeto IL, Domm BL, Curiel D, Lewis JH. Uremic bleeding. *Pennsylvania Med* 1969; 72: 65–69.
17 Singh G, Hussain SK, Matthai TP, Sharma SD, Chugh KS, Chhuttani PN. Hemostatic mechanism in uremia. *Ind J Med Sci*1969; 23: 387–394.
18 Losowsky MS, Walls WD. Abnormalities of haemostasis in renal failure. *J R Coll Physicians Lond* 1971; 5: 148–156.
19 Berkessy S, Faludi AG. The spreading ability of platelets in chronic uremia. *Ann Clin Res* 1974; 4: 65–67.
20 Panicucci F, Sagripanti A, Pinori E, Lecchini L, Barsotti G, Giovannetti S. Comprehensive study of haemostasis in chronic uremia. *Nephron* 1983; 33: 5–8.
21 Willoughby MLN, Crouch SJ. An investigation of the haemorrhagic tendency in renal failure. *Br J Haematol* 1961; 7: 315.
22 Castaldi PA, Rozenberg MC, Stewart JH. The bleeding disorder of uremia: a qualitative platelet defect. *Lancet* 1966; ii: 66–69.
23 Gafter U, Bessler H, Malachi T, Zevin D, Djaldetti M, Levi J. Platelet count and thrombopoietic activity in patients with chronic renal failure. *Nephron* 1987; 45: 207–210.
24 Hakim RM, Schafer AI. Hemodialysis-associated platelet activation and thrombocytopenia. *Am J Med* 1985; 78: 575–580.
25 Knudsen F, Nielsen A, Kristensen SD. The effect of dialyzer membrane material on intradialytic changes in platelet count, platelet aggregation, circulating platelet aggregates and antithrombin III. *Scand J Urol Nephrol* 1985; 19: 227–232.
26 Matsuo T, Yamada T, Yamanaehi T, Ryo R. Anticoagulant therapy with MD 805 of a hemodialysis patient with heparin-induced thrombocytopenia. *Thromb Res* 1990; 58: 663–666.
27 Hall AV, Clark WF, Parbtani A. Heparin-induced thrombocytopenia in renal failure. *Clin Nephrol* 1992; 38: 86–89.
28 Michalak E, Walkowiak B, Paradowski M, Cieriewski CS. The decreased circulating platelet mass and its relation to bleeding time in chronic renal failure. *Thromb Haemost* 1991; 65: 11–14.
29 DiMinno G, Martinez J, McKean ML, De La Rosa J, Burke JF, Murphy S. Platelet dysfunction in uremia. Multifaceted defect partially corrected by dialysis. *Am J Med* 1985; 79: 552–559.
30 Eknoyan G, Brown CH III. Biochemical abnormalities of platelets in renal failure. Evidence for decreased platelet serotonin, adenosine diphosphate and Mg-dependent adenosine triphosphatase. *Am J Nephrol* 1981; 1: 17–23.
31 Vlachoyannis J, Schoeppe W. Adenylate cyclase activity and cAMP content of human platelets in uremia. *Eur J Clin Invest* 1982; 12: 379–381.
32 Gura V, Creter D, Levi J. Elevated thrombocyte calcium content in uremia and its correction by alpha (OH) vitamin D treatment. *Nephron* 1982; 30: 237–239.
33 Ware JA, Clark BA, Smith M, Salzman EW. Abnormalities of cytoplasmic Ca^{2+} in platelets from patients with uremia. *Blood* 1989; 73: 172–176.
34 Remuzzi G, Benigni A, Dodesini P et al. Parathyroid hormone inhibits human platelet function. *Lancet* 1981; ii: 1321–1323.
35 Benigni A, Livio M, Dodesini P et al. Inhibition of human platelet aggregation by parathyroid hormone. Is cyclic AMP implicated? *Am J Nephrol* 1985; 5: 243–247.
36 Viganò G, Gotti E, Comberti E, Giangrande A, Trevisan R, Remuzzi G. Hyperparathyroidism does not influence the abnormal primary haemostasis in patients with chronic renal failure. *Nephrol Dial Transpl* 1989; 4: 971–974.
37 Kyrle PA, Stockenhuber F, Brenner B et al. Evidence for an

Cambridge Books Online © Cambridge University Press, 2009

increased generation of prostacyclin in the microvasculature and an impairment of platelet alpha-granule release in chronic renal failure. *Thromb Haemost* 1988; 60: 205–208.
38 Hutton RA, O'Shea MJ. Haemostatic mechanisms in uraemia. *J Clin Pathol* 1968; 21: 406–411.
39 Joist JH, Pechan J, Schikowski U, Hübner G, Gross R. Untersuchungenzur natur und atiologie der uramischen thrombocytopathie. *Verh Dtsch Ges Inn Med* 1969; 75: 476–479.
40 Salzman EW, Neri LL. Adhesiveness of blood platelets in uremia. *Thrombos Diathes Haemorrh* 1966; 15: 84–92.
41 Evans EP, Branch RA, Bloom AL. A clinical and experimental study of platelet function in chronic renal failure. *J Clin Path* 1972; 25: 745–753.
42 Schondoft TH, Hey D. Platelet function tests in uremia and under acetylsalicylic acid administration. *Haemostasis* 1974; 3: 129–136.
43 Turney JH, Woods HF, Fewell MR, Weston MJ. Factor VIII complex in uremia and effects of hemodialysis. *Br Med J* 1981; 282: 1653–1656.
44 Gordge MP, Dodd NJ, Rylance PB, Weston MJ. An assessment of whole blood impedance aggregometry using blood from normal subjects and hemodialysis patients. *Thromb Res* 1984; 36: 17–27.
45 Gordge MP, Faint RW, Rylance RB, Neild GH. Platelet function and the bleeding time in progressive renal failure. *Thromb Haemost* 1988; 60: 83–87.
46 Ballard HS, Marcus AJ. Primary and secondary platelet aggregation in uraemia. *Scand J Haematol* 1972; 9: 198–203.
47 Rylance PB, Gordge MP, Weston MJ. Platelet function is enhanced, not reduced in uremia. *Proc Eur Dial Transplant Ass* 1985; 22: 946.
48 Moosa A, Greaves M, Brown CB, MacNeil S. Elevated platelet-free calcium in uremia. *Br J Haematol* 1990; 74: 300–305.
49 Remuzzi G, Marchesi D, Livio M et al. Altered platelet and vascular prostaglandin-generation in patients with renal failure and prolonged bleeding times. *Thromb Res* 1978; 13: 1007–1015.
50 Smith MC, Dunn MJ. Impaired platelet thromboxane production in renal failure. *Nephron* 1981; 29: 133–137.
51 Remuzzi G, Benigni A, Dodesini P et al. Reduced platelet thromboxane formation in uremia. Evidence for a functional cyclo-oxygenase defect. *J Clin Invest* 1983; 71: 762–768.
52 Winter M, Frampton G, Bennett A, Machin SJ, Cameron JS. Synthesis of thromboxane B_2 in uraemia and the effects of dialysis. *Thromb Res* 1983; 30: 265–272.
53 Bloom A, Greaves M, Preston FE, Brown CB. Evidence against a platelet cyclooxygenase defect in uremia subjects on chronic haemodialysis. *Br J Hematol* 1986; 62: 143.
54 Remuzzi G, Livio M, Cavenaghi AE et al. Unbalanced prostaglandin synthesis and plasma factors in uremic bleeding. A hypothesis. *Thromb Res* 1978; 13: 531–536.
55 Weiss JH, Turitto VT, Baumgartner HR. Effect of shear rate in platelet interaction with subendothelium in citrated native blood. I. Shear rate-dependent decrease in adherence in von Willebrand's disease and the Bernard–Soulier syndrome. *J Lab Clin Med* 1978; 92: 750–764.
56 Sakariassen KS, Bolhuis PA, Sixma JJ. Platelet adherence to subendothelium of human arteries in pulsatile and steady flow. *Thromb Res* 1980; 19: 547–559.
57 Benigni A, Boccardo P, Galbusera M et al. Reversible activation defect of the platelet glycoprotein IIb–IIIa complex in patients with uremia. *Am J Kidney Dis* 1993; 22: 668–676.
58 Mezzano D, Tagle R, Panes O et al. Hemostatic disorder of uremia: the platelet defect, main determinant of the prolonged bleeding time, is correlated with indices of activation of coagulation and fibrinolysis. *Thromb Haemost* 1996; 76: 312–321.
59 Sloand EM, Sloand JA, Prodouz K et al. Reduction of platelet glycoprotein Ib in uraemia. *Br J Haematol* 1991; 77: 375–381.
60 Gawaz MP, Dobos G, Spath M, Schollmeyer P, Gurland HJ, Mujais SK. Impaired function of platelet membrane glycoprotein IIb–IIIa in end-stage renal disease. *J Am Soc Nephrol* 1994; 5: 36–46.
61 Sreedhara R, Itagaki I, Hakim RM. Uremic patients have decreased shear-induced platelet aggregation mediated by decreased availability of glycoprotein IIb–IIIa receptors. *Am J Kidney Dis* 1996; 27: 355–364.
62 Castillo R, Lozano T, Escolar G, Revert L, Lopez J, Ordinas A. Defective platelet adhesion on vessel subendothelium in uremic patients. *Blood* 1986; 65: 337–342.
63 Zwaginga JJ, Ijsseldijk MJW, Beeser-Visser N, de Groot PG, Vos J, Sixma JJ. High von Willebrand factor concentration compensates a relative adhesion defect in uremic blood. *Blood* 1990; 75: 1498–1508.
64 Escolar G, Cases A, Bastida E et al. Uremic platelets have a functional defect affecting the interation of von Willebrand factor with glycoprotein IIb–IIIa. *Blood* 1990; 76: 1336–1340.
65 Deykin D. Uremic bleeding. *Kidney Int* 1983; 24: 698–705.
66 Livio M, Mannucci PM, Vigano G et al. Conjugated estrogens for the management of bleeding associated with renal failure. *N Engl J Med* 1986; 315: 731–735.
67 Janson PA, Jubelirer SJ, Weinstein MJ, Deykin D. Treatment of the bleeding tendency in uremia with cryoprecipitate. *N Engl J Med* 1980; 303: 1318–1322.
68 Mannucci PM, Remuzzi G, Pusineri F et al. Deamino-8–D-arginine vasopressin shortens the bleeding time in uremia. *N Engl J Med* 1983; 308: 8–12.
69 Remuzzi G, Cavenaghi AE, Mecca G, Donati MB, de Gaetano G. Prostacyclin-like activity and bleeding in renal failure. *Lancet* 1977; ii: 1195–1197.
70 Silberbauer K, Sinzinger H, Winter M. Increased prostacyclin availability of vascular tissue in experimentally induced acute uremia in rats. *Artery* 1978; 4: 554.
71 Zoja C, Vigano G, Bergamelli A, Benigni A, de Gaetano G, Remuzzi G. Prolonged bleeding time and increased vascular prostacyclin in rats with chronic renal failure: effects of conjugated estrogens. *J Lab Clin Med* 1988; 112: 380–386.
72 Defreyn G, Dauden MV, Machin SJ, Vermylen J. A plasma

Cambridge Books Online © Cambridge University Press, 2009

factor in uraemia which stimulates prostacyclin release from cultured endothelial cells. *Thromb Res* 1980; 19: 695.

73 Radomski MW, Palmer RM, Moncada S. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet* 1987; ii: 1057–1058.

74 Radomski MW, Palmer RM, Moncada S. An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc Natl Acad Sci USA* 1990; 87: 5193–5197.

75 Neri I, Marietta M, Piccinini F, Volpe A, Facchinetti F. The L-arginine-nitric oxide system regulates platelet aggregation in pregnancy. *J Soc Gynecol Invest* 1998; 5: 192–196.

76 Marietta M, Facchinetti F, Neri I, Piccinini F, Volpe A, Torelli G. L-arginine infusion decreases platelet aggregation through an intraplatelet nitric oxide release. *Thromb Res* 1997; 88: 229–235.

77 Noris M, Benigni A, Boccardo P et al. Enhanced nitric oxide synthesis in uremia: implications for platelet dysfunction and dialysis hypotension. *Kidney Int* 1993; 44: 445–450.

78 Noris M, Remuzzi G. Uremic bleeding: closing the circle after 30 years of controversies? *Blood* 1999; 94: 2569–2574.

79 Matsumoto A, Hirata Y, Kakoki, M et al. Increased excretion of nitric oxide in exhaled air of patients with chronic renal failure. *Clin Sci* 1999; 96: 67–74.

80 Madore F, Prud'homme L, Austin JS et al. Impact of nitric oxide on blood pressure in hemodialysis patients. *Am J Kidney Dis* 1997; 30: 665–671.

81 Yokokawa K, Kohno M, Yoshikawa J. Nitric oxide mediates the cardiovascular instability of haemodialysis patients. *Curr Opin Nephrol Hyperten* 1996; 5: 359–363.

82 Yokokawa K, Mankus R, Saklayen MG et al. Increased nitric oxide production in patients with hypotension during hemodialysis. *Ann Intern Med* 1995; 123: 35–37.

83 Martensson L, Hegbrandt J, Thysell H. Generation of nitrate during dialysis as a measure of nitric oxide synthesis. *Artif Organs* 1997; 21: 163–167.

84 Thuraisingham RC, Cramp HA, McMahon AC et al. Increased superoxide and nitric oxide production results in peroxynitrite formation in uremic vasculature. *J Am Soc Nephrol* 1997; 8: 340A. (abstract)

85 Descamps-Latscha B, Herbelin A, Nguyen AT et al. Balance between IL-1β, TNF-α, and their specific inhibitors in chronic renal failure and maintenance dialysis. Relationships with activation markers of T cells, B cells, and monocytes. *J Immunol* 1995; 154: 882–892.

86 Herbelin A, Nguyen AT, Zingraff J, Urena P, Descamps-Latscha B. Influence of uremia and hemodialysis on circulating interleukin-1 and tumor necrosis factor alpha. *Kidney Int* 1990; 37: 116–125.

87 Pereira BJG, Dinarello CA. Production of cytokines and cytokine inhibitory proteins in patients on dialysis. *Nephrol Dial Transpl* 1994; 9: 60–71.

88 Luger A, Kovarik J, Stummvoll HK, Urbanska A, Luger TA. Blood-membrane interaction in hemodialysis leads to increased cytokine production. *Kidney Int* 1987; 32: 84–88.

89 Rabiner SF, Molinas F. The role of phenol and phenolic acids on the thrombocytopathy and defective platelet aggregation of patients with renal failure. *Am J Med* 1970; 49: 346–351.

90 Horowitz HI, Stein IM, Cohen BD, White JG. Further studies on the platelet inhibitory effect of guanidinosuccinic acid and its role in uremic bleeding. *Am J Med* 1970; 49: 336–345.

91 Remuzzi G, Livio E, Marchiaro G, Mecca G, de Gaetano G. Bleeding in renal failure: Altered platelet function in chronic uraemia only partially corrected by haemodialysis. *Nephron* 1978; 22: 347–353.

92 Stein IM, Cohen BD, Horowitz HI. Guanidinosuccinic acid: the 'X' factor in uremic bleeding? *Clin Res* 1968; 16: 397 (abstract).

93 Horowitz HI. Uremic toxins and platelet function. *Arch Intern Med* 1970; 126: 823–826.

94 Sloand EM, Bern MM, Kaldany A. Effect on platelet function of hypoalbuminemia in peritoneal dialysis. *Thromb Res* 1986; 44: 419–425.

95 Turitto VT, Weiss HJ. Red blood cells: their dual role in thrombus formation. *Science* 1980; 207: 541–543.

96 Gaarder A, Jonsen J, Lland S et al. Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. *Nature* 1961; 192: 531–532.

97 Willems C, Stel HV, van Aken WG, Van Novrik JA. Binding and inactivation of prostacyclin (PGI_2) by human erythrocytes. *Br J Hematol* 1983; 54: 43–52.

98 Livio M, Gotti E, Marchesi, D, Mecca G, Remuzzi G, de Gaetano G. Uraemic bleeding: Role of anemia and beneficial effect of red cell transfusions. *Lancet* 1982; ii: 1013–1015.

99 Fernandez F, Goudable C, Sie P et al. Low hematocrit and prolonged bleeding time in uraemic patients: Effect of red cell transfusions. *Br J Hematol* 1985; 59: 139–148.

100 Howard AD, Moore J Jr, Welch PG, Gouge SF. Analysis of the quantitative relationship between anemia and chronic renal failure. *Am J Med Sci* 1989; 297: 309–313.

101 Eschbach JW, Adamson JW. Recombinant human erythropoietin: implications for nephrology. *Am J Kidney Dis* 1988; 11: 203–209.

102 Van Geet C, Hauglustaine D, Verresen L, Vanrusselt M, Vermylen J. Haemostatic effects of recombinant human erythropoietin in chronic haemodialysis patients. *Thromb Haemost* 1989; 61: 117–121.

103 Zwaginga JJ, Ijsseldijk MJ, de Groot PG et al. Treatment of uremic anemia with recombinant erythropoietin also reduces the defects in platelet adhesion and aggregation caused by uremic plasma. *Thromb Haemost* 1991; 66: 638–647.

104 NKF-DOQI. Clinical practice guidelines: treatment of anemia of chronic renal failure. *Am J Kidney Dis* 1997; 30 (Suppl.3): S192–S237.

105 Besarab A, Bolton WK, Browne JK et al. The effects of normal as compared with low hematocrit values in patients with cardiac disease who are receiving hemodialysis and epoetin. *N Engl J Med* 1998; 339: 584–590.

106 Minetti L. Erythropoietin treatment in renal anemia: how high should the target hematocrit be? *J Nephrol* 1997; 10: 117–119.

Cambridge Books Online © Cambridge University Press, 2009

107 Andrassy K, Ritz E. Uremia as a cause of bleeding. *Am J Nephrol* 1985; 5: 313–319.
108 Fass RJ, Copelan EA, Brandt JT, Moeschberger ML, Ashton JJ. Platelet-mediated bleeding caused by broad-spectrum penicillins. *J Infect Dis* 1987; 155: 1242–1248.
109 Shattil SJ, Bennett JS, McDonough M, Turnbull J. Carbenicillin and penicillin G inhibit platelet function in vitro by impairing the interaction of agonists with the platelets surface. *J Clin Invest* 1980; 65: 329–337.
110 Bang N, Tessler S, Heidenreich R, Marks C. Effects of moxolactan on blood coagulation and platelet function. *Rev Infect Dis* 1982; 4: S546–S554.
111 Bechtold H, Andrassy K, Jahnchen E et al. Evidence for impaired hepatic vitamin K metabolism in patients treated with N-methyl-thiotetrazole cephalosporin. *Thromb Haemost* 1984; 51: 358–361.
112 Harter HR, Burch JW, Majerus PW et al. Prevention of thrombosis in patients on hemodialysis by low dose of aspirin. *N Engl J Med* 1979; 301: 577–579.
113 Lindsay RM, Ferguson D, Prentice CR, Burton JA, McNicol GP. Reduction of thrombus formation on dialyser membranes by aspirin and RA233. *Lancet* 1972; ii: 1287–1290.
114 Livio M, Benigni A, Viganò G, Mecca G, Remuzzi G. Moderate doses of aspirin and risk of bleeding in renal failure. *Lancet* 1986; i: 414–416.
115 Gaspari F, Viganò G, Orisio S, Bonati M, Livio M, Remuzzi G. Aspirin prolongs bleeding time in uremia by a mechanism distinct from platelet cyclooxygenase inhibition. *J Clin Invest* 1987; 79: 1788–1797.
116 Zuckerman GR, Cornette GL, Clouse RE, Harter HR. Upper gastrointestinal bleeding in patients with chronic renal failure. *Ann Intern Med* 1985; 102: 588–592.
117 Boyle JM, Johnston B. Acute upper gastrointestinal hemorrhage in patients with chronic renal disease. *Am J Med* 1983; 75: 409–412.
118 Simon SL, Mills JA. Non-steroidal anti-inflammatory drugs. *N Engl J Med* 1980; 302: 1337–1343.
119 Harker LA, Fuster V. Pharmacology of platelet inhibitors. *J Am Coll Cardiol* 1986; 8: 21B–32B.
120 Lazarus JM, Hakim RM. Medical aspects of hemodialysis. In Brenner BM, Rector FC eds, *The kidney*. Philadelphia: WB Saunders; 1991: 2223–2298.
121 Hammel FS, Johnson GC, Collins GM. A critical appraisal of the changing approaches to vascular access for chronic dialysis. *Proc Eur Dial Transplant Assoc* 1982; 19: 325–330.
122 Kaegi A, Pineo GF, Shimizu A, Trivedi H, Hirsh J, Gent M. Arteriovenous shunt thrombosis. Prevention by sulfinpyrazone. *N Engl J Med* 1974; 290: 304–306.
123 Michie DD, Wombolt DG. Use of sulfinpyrazone to prevent thrombus formation in arteriovenous fistulas and bovine grafts of patients on chronic hemodialysis. *Ther Res* 1977; 22: 196.
124 Kobayashi K, Maeda K, Koshikawa S, Kawaguchi Y, Shimizu N, Naito C. Antithrombotic therapy with ticlopidine in chronic renal failure patients on maintenance hemodialysis: a multicenter collaborative double-blind study. *Thromb Res* 1980; 20: 255–261.
125 Fiskerstrand CE, Thompson IW, Burnet ME, Williams P, Anderton JL. Double-blind randomized trial of the effect of ticlopidine in arteriovenous fistulas for hemodialysis. *Artif Organs* 1985; 9: 61–63.
126 Gröntoft KC, Mulec H, Gutierrez A, Olander R. Thromboprophylactic effect of ticlopidine in arteriovenous fistulas for haemodialysis. *Scand J Urol Nephrol* 1985; 19: 55.
127 Avram MM. Cholesterol and lipids in renal disease. *Am J Med* 1989; 87: 1N–2N.
128 Joist JH, Remuzzi G, Mannucci PM. Abnormal bleeding and thrombosis in renal disease. In Colman RW, Hirsh J, Marde VJ, Salzman EW eds. *Hemostasis and thrombosis: basic principles and clinical practice*. Philadelphia: JB Lippincott Company; 1994: 921–935.
129 Cameron JS. Coagulation and thromboembolic complications in the nephrotic syndrome. *Adv Nephrol* 1984; 13: 75–114.
130 Llach F. Hypercoagulability, renal vein thrombosis, and other thrombotic complications of nephrotic syndrome. *Kidney Int* 1985; 28: 429–439.
131 Barnett HL. Minimal change nephrotic syndrome in children: deaths during the first 5–15 years' observation. Report of the International Study of Kidney Disease in Children. *Pediatrics* 1984; 73: 497.
132 Mehls O, Andrassy K, Koderisch J, Herzog U, Ritz E. Hemostasis and thromboembolism in children with nephrotic syndrome: differences from adults. *J Pediatr* 1987; 110: 862–867.
133 Anderson S, Garcia DL, Brenner BM. Renal and systemic manifestations of glomerular disease. In Brenner BM, Rector FCJr eds, *The kidney*. Philadelphia: WB Saunders; 1991: 1831–1870.
134 Kendall AG, Lohmann RC, Dossetor JB. Nephrotic syndrome. A hypercoagulable state. *Arch Intern Med* 1971; 127: 1021–1027.
135 Bang NU, Trygstad W, Schroeder JE, Heidenreich RO, Csiscko BM. Enhanced platelet function in glomerular renal disease. *J Lab Clin Med* 1973; 81: 651–660.
136 Schieppati A, Dodesini P, Benigni A et al. The metabolism of arachidonic acid by platelets in nephrotic syndrome. *Kidney Int* 1984; 25: 671–676.
137 Robert A, Olmer M, Sampol J, Gugliotta JE, Casanova P. Clinical correlation between hypercoagulability and thromboembolic phenomena. *Kidney Int* 1987; 31: 830–835.
138 Bennett A, Cameron JS. Platelet hyperaggregability in the nephrotic syndrome which is not dependent on arachidonic acid metabolism or on plasma albumin concentration. *Clin Nephrol* 1987; 27: 182–188.
139 Elidrissy AT, Abdurrahman MB, Bahakim HM, Jones MD, Gader AM. Haemostatic measurements in childhood nephrotic syndrome. *Eur J Pediatr* 1991; 150: 374–378.
140 Andrassy K, Ritz E, Bommer J. Hypercoagulability in the nephrotic syndrome. *Klin Wochenschr* 1980; 58: 1029–1036.

Cambridge Books Online © Cambridge University Press, 2009

141 Stuart MJ, Spitzer RE, Nelson DA, Sills RH. Nephrotic syndrome: Increased platelet prostaglandin endoperoxide formation, hyperaggregability, and reduced platelet life span. Reversal following remission. *Pediatr Res* 1980; 14: 1078–1081.
142 Walter E, Deppermann D, Andrassy K, Koderisch J. Platelet hyperaggregability as a consequence of the nephrotic syndrome. *Thromb Res* 1981; 23: 473–479.
143 Cameron JS. Platelets in glomerular disease. *Ann Rev Med* 1984; 35: 175–180.
144 Zoja C, Livio M, Benigni A, Remuzzi G. Eicosanoids and platelet activating factor as mediators of injury in renal disease. In Remuzzi G, Rossi EC eds, *Haemostasis and the kidney*. London: Butterworths; 1989: 92–104.
145 Carruthers JA, Ralfs I, Gimlette TM, Finn R. Platelet survival in acute proliferative glomerulonephritis. *Clin Sci Mol Med* 1974; 47: 507–513.
146 Clark WF, Lewis ML, Cameron JS, Parsons V. Intrarenal platelet consumption in the diffuse proliferative nephritis of systemic lupus erythematosus. *Clin Sci Mol Med* 1975; 49: 247–252.
147 George CRP, Slichter SJ, Quadracci LJ, Striker GE, Harker LA. A kinetic evaluation of hemostasis in renal disease. *N Engl J Med* 1974; 291: 1111–1115.
148 Parbtani A, Clark WF, Cameron JS. Pathogenic significance of platelet activation in lupus nephritis. *Transpl Proc* 1986; 18: 657–658.
149 Kant KS, Pollak VE, Weiss MA, Glueck HI, Miller MA, Hess EV. Glomerular thrombosis in systemic lupus erythematosus: prevalence and significance. *Medicine* 1981; 60: 71–86.
150 Heptinstall RH. Hemolytic uremic syndrome, thrombotic, thrombocytopenic purpura, and systemic sclerosis (systemic scleroderma). In Heptinstall RH, ed., *Pathology of the kidney*. Boston: Little, Brown and Co.; 1992: 1163–1233.
151 Amenta PS, Katz SM. Platelets in renal scleroderma. *Arch Pathol Lab Med* 1983; 107: 439–440.
152 Porter KA. Renal transplantation. In Heptinstall RH, ed., *Pathology of the kidney*. Boston: Little, Brown and Co.; 1992: 1799–1933.
153 Heptinstall RH. Hypertension I: Essential hypertension. In Heptinstall RH, ed., *Pathology of the kidney*. Boston: Little, Brown and Co.; 1992: 951–1028.
154 Duffy JL, Cinque T, Grishman E, Churg J. Intraglomerular fibrin, platelet aggregation, and subendothelial deposits in lipoid nephrosis. *J Clin Invest* 1970; 49: 251–258.
155 Kincaid-Smith P, Laver MC, Fairley KF. Dipyridamole and anticoagulants in renal disease due to glomerular and vascular lesions. A new approach to therapy. *Med J Aust* 1970; 1: 145–151.
156 Nakajima M, Hewitson TD, Mathews DC, Kincaid-Smith P. Platelet-derived growth factor mesangial deposits in mesangial IgA glomerulonephritis. *Nephrol Dial Transpl* 1991; 6: 11–16.
157 Woo KT, Junor BJ, Salem H, d'Apice AJ, Whitworth JA, Kincaid-Smith P. Beta-thromboglobulin and platelet aggregates in glomerulonephritis. *Clin Nephrol* 1980; 14: 92–95.
158 Duffus P, Parbtani A, Frampton G, Cameron JS. Intraglomerular localization of platelet related antigens, platelet factor 4 and β-thromboglobulin in glomerulonephritis. *Clin Nephrol* 1982; 17: 288–297.
159 Schlondorff D, Neuwirth P. Platelet activating factor and the kidney. *Am J Physiol* 1986; 251: F1–F11.
160 Camussi G. Potential role of platelet-activating factor in renal pathophysiology. *Kidney Int* 1986; 29: 469–477.
161 Braquet P, Touqui L, Shen TY, Vargaftig BB. Perspectives in platelet-activating factor research. *Pharmacol Rev* 1987; 39: 97–145.
162 Lianos EA, Zanglis A. Glomerular platelet-activating factor levels and origin in experimental glomerulonephritis. *Kidney Int* 1990; 37: 736–740.
163 Bertani T, Livio M, Macconi D et al. Platelet activating factor (PAF) as a mediator of injury in nephrotoxic nephritis. *Kidney Int* 1987; 31: 1248–1256.
164 Yoo J, Schlondorff D, Neugarten J. Protective effects of specific platelet-activating factor receptor antagonists in experimental glomerulonephritis. *J Pharmacol Exp Ther* 1991; 256: 841–844.
165 Camussi G, Tetta C, Deregitus C, Bussolino F, Segoloni G, Vercellone A. Platelet-activating factor (PAF) in experimentally-induced rabbit acute serum sickness: role of basophil-derived PAF in immune complex deposition. *J Immunol* 1982; 128: 86–94.
166 Stahl RAK, Thaiss F, Oberle G et al. The platelet activating factor receptor antagonist WEB 2170 improves glomerular hemodynamics and morphology in a proliferative model of mesangial cell injury. *J Am Soc Nephrol* 1991; 2: 37–44.
167 Baldi E, Emancipator SN, Hassan MO, Dunn MJ. Platelet activating factor receptor blockade ameliorates murine systemic lupus erythematosus. *Kidney Int* 1990; 38: 1030–1038.
168 Morigi M, Macconi D, Riccardi E et al. Platelet-activating factor receptor blocking reduces proteinuria and improves survival in lupus autoimmune mice. *J Pharmacol Exp Ther* 1991; 258: 601–606.
169 Macconi D, Noris M, Benfenati E, Quaglia R, Pagliarino G, Remuzzi G. Increased urinary excretion of platelet activating factor in mice with lupus nephritis. *Life Sci* 1991; 48: 1429–1437.
170 Barnes JL, Levine SP, Venkatachalam MA. Binding of platelet factor four (PF 4) to glomerular polyanion. *Kidney Int* 1984; 25: 759–765.
171 Tetta C, Coda R, Camussi G. Human platelet cationic proteins bind to rat glomeruli, induce loss of anionic charges and increase glomerular permeability. *Agents Actions* 1985; 16: 24–26.
172 Camussi G, Tetta C, Coda R, Segoloni GP, Vercellone A. Platelet-activating factor-induced loss of glomerular anionic charges. *Kidney Int* 1984; 25: 73–81.
173 Barnes JL, Camussi G, Tetta C, Venkatachalam MA. Glomerular localization of platelet cationic proteins after immune complex-induced platelet activation. *Lab Invest* 1990; 63: 755–761.

Cambridge Books Online © Cambridge University Press, 2009

174 Camussi G, Tetta C, Mazzucco G et al. Platelet cationic proteins are present in glomeruli of lupus nephritis patients. *Kidney Int* 1986; 30: 555–565.
175 Camussi G, Tetta C, Segoloni G, Coda R, Vercellone A. Localization of neutrophil cationic proteins and loss of anionic charges in glomeruli of patients with systemic lupus erythematosus glomerulonephritis. *Clin Immunol Immunopathol* 1982; 24: 299–314.
176 Barnes JL. Platelets in renal disease. In Tetta C, ed. *Immunopharmacology of the renal system. Handbook of immunopharmacology*. London: Academic Press; 1993; 88–118.
177 Abboud HE. Growth factors in glomerulonephritis. *Kidney Int* 1993; 43: 252–267.
178 Johnson R, Iida H, Yoshimura A, Floege J, Bowen-Pope DF. Platelet-derived growth factor: a potentially important cytokine in glomerular disease. *Kidney Int* 1992; 41: 590–594.
179 Johnson RJ. Platelets in inflammatory glomerular injury. *Semin Nephrol* 1991; 11: 276–284.
180 Shultz PJ, DiCorleto PE, Silver BJ, Abboud HE. Mesangial cells express PDGF mRNAs and proliferate in response to PDGF. *Am J Physiol* 1988; 255: F674–F684.
181 Silver BJ, Jaffer FE, Abboud HE. Platelet-derived growth factor synthesis in mesangial cells: induction by multiple peptide mitogens. *Proc Natl Acad Sci USA* 1989; 86: 1056–1060.
182 Abboud HE. Platelet-derived growth factor and mesangial cells. *Kidney Int* 1992; 41: 581–583.
183 Barnes JL, Hevey KA. Glomerular mesangial cell migration. Response to platelet secretory products. *Am J Pathol* 1991; 138: 859–866.
184 Taraboletti G, Morigi M, Figliuzzi M, Giavazzi R, Zoja C, Remuzzi G. Thrombospondin induces glomerular mesangial cell adhesion and migration. *Lab Invest* 1992; 67: 566–571.
185 Johnson RJ, Raines EW, Floege J et al. Inhibition of mesangial cell proliferation and matrix expansion in glomerulonephritis in the rat by antibody to platelet-derived growth factor. *J Exp Med* 1992; 175: 1413–1416.
186 Iida H, Seifert R, Alpers CE et al. Platelet-derived growth factor (PDGF) and PDGF receptor are induced in mesangial proliferative nephritis in the rat. *Proc Natl Acad Sci USA* 1991; 88: 6560–6564.
187 Floege J, Burns MW, Alpers CE et al. Glomerular cell proliferation and PDGF expression precede glomerulosclerosis in the remnant kidney model. *Kidney Int* 1992; 41: 297–309.
188 Young BA, Johnson RJ, Alpers CE et al. Cellular events in the evolution of experimental diabetic nephropathy. *Kidney Int* 1995; 47: 935–944.
189 Gesualdo L, Pinzani M, Floriano JJ et al. Platelet-derived growth factor expression in mesangial proliferative glomerulonephritis. *Lab Invest* 1991; 65: 160–167.
190 Gesualdo L, Di Paolo S, Milani S et al. Expression of platelet-derived growth factor receptors in normal and diseased human kidney. An immunohistochemistry and in situ-hybridization study. *J Clin Invest* 1994; 94: 50–58.
191 Rubin K, Tingstrom A, Hasson GK et al. Induction of β-type receptors for platelet-derived growth factor in vascular inflammation: possible implications for development of vascular proliferative lesions. *Lancet* 1988; i: 1353–1356.
192 Fellström B, Klareskog L, Heldin CH et al. Platelet-derived growth factor receptors in the kidney. Upregulated expressions in inflammation. *Kidney Int* 1989; 36: 1099–1102.
193 Border WA, Ruoslahti E. Transforming growth factor-β in disease: the dark side of tissue repair. *J Clin Invest* 1992; 90: 1–7.
194 Wahl SM. Transforming growth factor-β: the good, the bad, and the ugly. *J Exp Med* 1994; 180: 1587–1590.
195 Border WA, Noble NA. Transforming growth factor-β in tissue fibrosis. *N Engl J Med* 1994; 331: 1286–1292.
196 Roberts AB, Sporn MB. The transforming growth factor-β. In Sporn MB, Roberts AB, eds. *Handbook of experimental pharmacology, peptide growth factors and their receptors.* Berlin, Heidelberg, New York: Springer; 1990: 419.
197 Jaffer F, Saunders C, Shultz P, Throckmorton D, Weinshell E, Abboud HE. Regulation of mesangial cell growth by polypeptide mitogens. Inhibitory role of transforming growth factor beta. *Am J Pathol* 1989; 135: 261–269.
198 MacKay K, Striker LJ, Stauffer JW, Doi T, Agodoa LY, Striker GE. Transforming growth factor-β. Murine glomerular receptors and responses of isolated glomerular cells. *J Clin Invest* 1989; 83: 1160–1167.
199 Massague J. The transforming growth factor-β family. *Annu Rev Cell Biol* 1990; 6: 597–641.
200 Okuda S, Languino LR, Ruoslahti E, Border WA. Elevated expression of transforming growth factor-β and proteoglycan production in experimental glomerulonephritis. Possible role in expansion of the mesangial extracellular matrix. *J Clin Invest* 1990; 86: 453–462.
201 Yamamoto T, Nakamura T, Noble NA, Ruoslahti E, Border WA. Expression of transforming growth factor β is elevated in human and experimental diabetic nephropathy. *Proc Natl Acad Sci USA* 1993; 90: 1814–1818.
202 Tamaki K, Okuda S, Ando T, Iwamoto T, Nakayama M, Fujishima M. TGF-β1 in glomerulosclerosis and interstitial fibrosis of adriamycin nephropathy. *Kidney Int* 1994; 45: 525–536.
203 Johnson RJ, Alpers CE, Pritzl P et al. Platelets mediate neutrophil-dependent immune complex nephritis in the rat. *J Clin Invest* 1988; 82: 1225–1235.
204 Johnson RJ, Alpers CE, Pruchno C et al. Mechanisms and kinetics for platelet and neutrophil localization in immune complex nephritis. *Kidney Int* 1989; 36: 780–789.
205 Kniker WT, Cochrane CG. The localization of circulating immune complexes in experimental serum sickness: The role of vasoactive amines and hydrodynamic forces. *J Exp Med* 1968; 127: 119–136.
206 Cattell V. Focal mesangial proliferative glomerulonephritis in the rat caused by Habu snake venom: the role of platelets. *Br J Exp Pathol* 1979; 60: 201–208.
207 Barnes JL, Hevey KA, Hastings RR, Bocanegra RA. Mesangial cell migration precedes proliferation in Habu snake venom-induced glomerular injury. *Lab Invest* 1994; 70: 460–467.
208 Johnson RJ, Garcia RL, Pritzl P, Alpers CE. Platelets mediate

Cambridge Books Online © Cambridge University Press, 2009

glomerular cell proliferation in immune complex nephritis induced by anti-mesangial cell antibodies in the rat. *Am J Pathol* 1990; 136: 369–374.
209 Kincaid-Smith P. The treatment of chronic mesangiocapillary (membranoproliferative) glomerulonephritis with impaired renal function. *Med J Aust* 1972; 2: 587–592.
210 Cattran D, Charron R, Cardella C. Controlled trial in mesangio-capillary glomerulonephritis (MCGN) (Abstract). In Zurukzoglu W, Papadimitriou M, Sion M, eds. *Eighth international congress of nephrology: advances in basic and clinical nephrology*. Basel: Karger; 1981: 287.
211 Zimmerman SW, Moorthy AV, Dreher WH, Friedman A, Varanasi U. Prospective trial of warfarin and dipyridamole in patients with membranoproliferative glomerulonephritis. *Am J Med* 1983; 75: 920–927.
212 Donadio JV Jr, Anderson CF, Mitchell JC III et al. Membranoproliferative glomerulonephritis. A prospective clinical trial of platelet-inhibitor therapy. *N Engl J Med* 1984; 310: 1421–1426.
213 McTavish D, Faulds D, Gao KL. Ticlopidine. An updated review of its pharmacology and therapeutic use in platelet-dependent disorders. *Drugs* 1990; 40: 238–259.
214 Hardisty RM, Powling MJ, Nokes TJC. The action of ticlopidine on human platelets. Studies on aggregation, secretion, calcium mobilization and membrane glycoproteins. *Thromb Haemost* 1990; 64: 150–155.
215 Grekas D, Alivanis P, Kalekou H, Syrganis C, Tourkantonis A. Are antiplatelet agents of value in the treatment of chronic glomerular disease? *Nephrol Dial Transplant* 1987; 2: 377–379.
216 Bennett CL, Kiss JE, Weinberg PD et al. Thrombotic thrombocytopenic purpura after stenting and ticlopidine. *Lancet* 1998; 352: 1036–1037.
217 Page Y, Tardy B, Zeni F, Comtet C, Terrana R, Bertrand JC. Thrombotic thrombocytopenic purpura related to ticlopidine. *Lancet* 1991; 337: 774–776.
218 Leavey SF, Weinberg J. Thrombotic thrombocytopenic purpura associated with ticlopidine therapy. *J Am Soc Nephrol* 1997; 8: 689–693.
219 Bennett CL, Weinberg PD, Rozenberg-Ben-Dror K, Yarnold PR, Kwaan HC, Green D. Thrombotic thrombocytopenic purpura associated with ticlopidine: a review of 60 cases. *Ann Intern Med* 1998; 128: 541–544.
220 Chen DK, Kim JS, Sutton DM. Thrombotic thrombocytopenic purpura associated with ticlopidine use. A report of 3 cases and review of the literature. *Arch Intern Med* 1999; 159: 311–314.
221 Moncada S, Higgs EA. Arachidonate metabolism in blood cells and the vessel wall. *Clin Haematol* 1986; 15: 273–292.
222 Leaf A, Weber PC. Cardiovascular effects of ω-3 fatty acids. *N Engl J Med* 1988; 318: 549–557.
223 Hamazaki T, Tateno S, Shishido H. Eicosapentaenoic and IgA nephropathy. *Lancet* 1984; i: 1017–1018.
224 Bennett WM, Walker RG, Kincaid-Smith P. Treatment of IgA nephropathy with eicosapentaenoic acid (EPA): a 2-year prospective trial. *Clin Nephrol* 1989; 31: 128–131.
225 Cheng IK, Chan PC, Chan MK. The effects of fish oil dietary supplement on the progression of mesangial IgA glomerulonephritis. *Nephrol Dial Transpl* 1990; 5: 241–246.
226 Pettersson EE, Rekola S, Berglund L et al. Treatment of IgA nephropathy with omega-3-polyunsaturated fatty acids: a prospective, double-blind, randomized study. *Clin Nephrol* 1994; 41: 183–190.
227 Donadio JV, Bergstralh EJ, Offord KP, Spencer DC, Holley KE. A controlled trial of fish oil in IgA nephropathy. Mayo nephrology collaborative group. *N Engl J Med* 1994; 331: 1194–1199.
228 Dillon JJ. Fish oil therapy for IgA nephropathy: efficacy and interstudy variability. *J Am Soc Nephrol* 1997; 8: 1739–1744.

Cambridge Books Online © Cambridge University Press, 2009

Platelets and allergic diseases

Simon Pitchford and Clive P. Page

Sackler Institute of Pulmonary Pharmacology, GKT School of Biomedical Sciences, Guy's Campus, King's College London, UK

Introduction

Irrefutable clinical evidence exists demonstrating the involvement of platelets in allergic diseases, including asthma, allergic rhinitis and eczema. Experimental models of allergic disease suggest that platelets may play a fundamental role in the pathogenesis of the inflammatory response that follows exposure to allergens. Of importance are the observations that, despite being anucleate, platelets share many characteristics of inflammatory cells and, of particular interest in allergy, undergo chemotaxis, express adhesion molecules, release a wide variety of proinflammatory mediators, enzymes, cationic proteins, and themselves become activated by mediators released by other cell types involved in inflammation.

Clinical evidence of platelet involvement in allergic diseases

Numerous studies have revealed an alteration in the character and function of platelets from patients with allergic diseases, and these alterations may be dissociated from the well-characterized involvement of platelets in thrombosis and hemostasis[1–7], illustrating a dichotomy in platelet fu[illegible]

platelet activation in vivo, whilst platelets from the same allergic patients are found to be refractory to a variety of stimuli ex vivo, a phenomenon possibly resulting from platelet 'exhaustion', where platelets are over stimulated in vivo and subsequently respond poorly to stimuli in vitro[8,9]. In support of this concept, platelet aggregation has been observed to be abnormal in asthmatic patients. The ability of proaggregatory mediators such as noradrenaline and adenosine diphosphate (ADP) to induce aggregation is seen to be impaired, with no second phase aggregation, an occurrence that has also been correlated with increased serum immunoglobulin E (IgE) in asthmatic patients[10,11]. It is also accompanied by a defective release of 5-hydroxytryptamine (5-HT) and platelet factor-4 (PF4)[10]. In vitro data investigating platelet function from atopic patients suffering from allergy to food, eczema, asthma or allergic rhinitis showed 5-HT uptake into platelets was slower compared to non-atopics. The release of 5-HT from washed platelets after stimulation with aggregated IgG was also significantly lower in the atopic groups[12], even though serum from asthmatic patients has been found to contain higher levels of 5-HT and its metabolites[13], thus raising the possibility of platelet desensitization due to chronic in vivo activation. The antidepressant drug tianeptine, a drug capable of reducing the levels of free 5-HT in plasma, via the enhancement of 5-HT uptake by platelets, resulted in a reduction in clinical severity of asthmatic indices in asthmatic patients, accompanied by an improvement in pulmonary function[14]. Other abnormalities of platelet function observed in vitro include a decrease in arachidonic acid metabolism in washed platelets from atopic subjects, although an increase in lipoxygenase products has been observed[15,16]. Platelet-activating factor (PAF) levels have been observed to rise during periods of acute asthma attacks. However, platelets from these patients [illegible] vitro[17]. Other investigations have suggested that intracellular second messenger systems are altered in platelets from atopic patients. Phosphatidyl inositol (PI) turnover and the formation of inositol triphosphate (IP_{145}) and intracellular Ca^{2+} $[Ca^{2+}]_i$ are found to be greater than basal levels, and are also heightened after in vitro exposure to PAF[18], providing evidence of increased in vivo stimulation.

These traits are accompanied by a prolonged bleeding time, increased platelet mass, an altered sensitivity to PAF of platelets isolated from patients with asthma and hay

Cambridge Books Online © Cambridge University Press, 2009

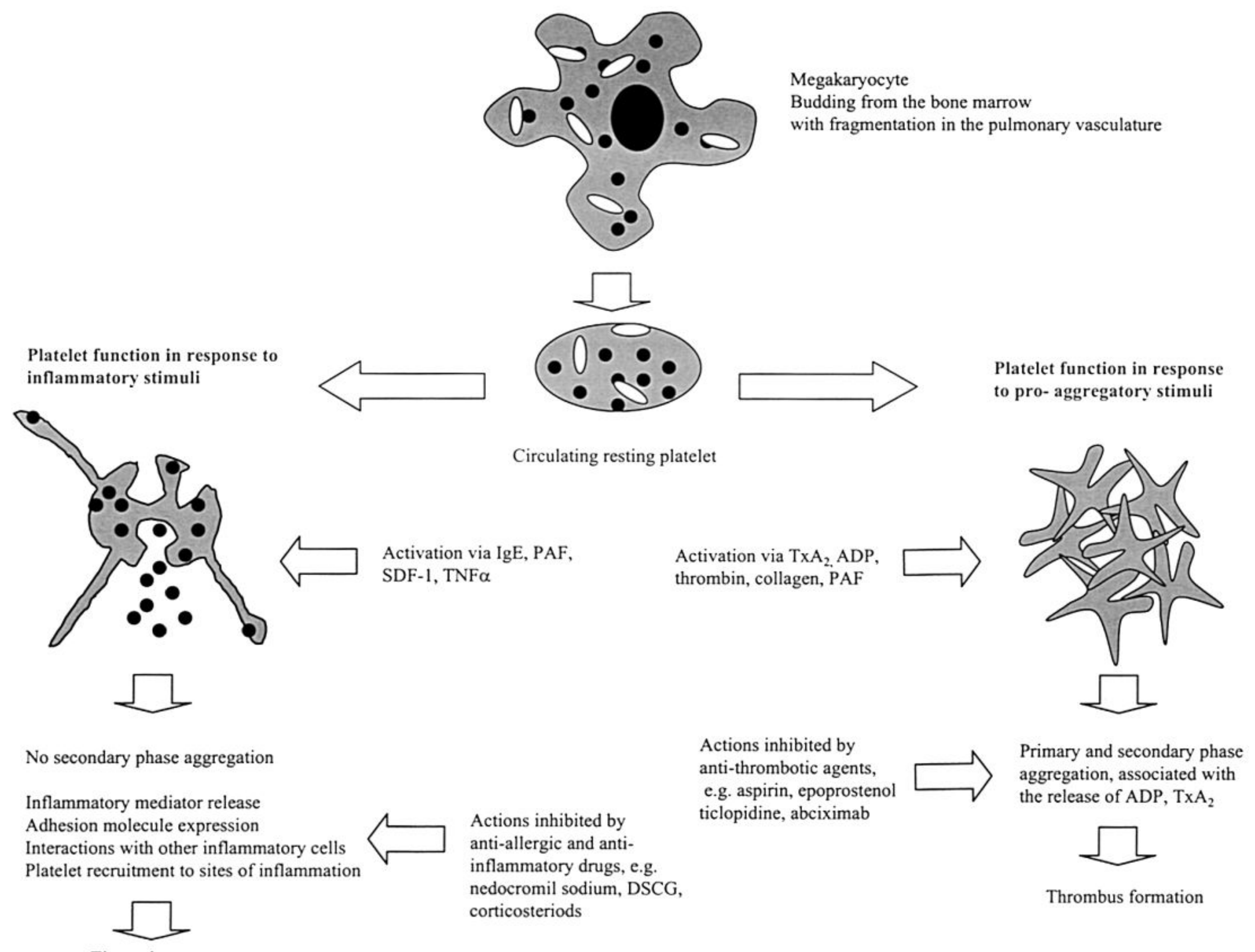


Fig. 56.1. Dichotomy of platelet function.

Cambridge Books Online © Cambridge University Press, 2009

fever, and an increase in platelet volume[16,19]. Observations have also been made of decreased platelet survival time in stable atopic asthmatics, perhaps as a result of chronic platelet activation[20]. These phenomena can be corrected by treatment of patients with glucocorticoids[21] or with platelets treated with disodium chromoglycate in vitro before reinfusion[22]. Large numbers of pulmonary megakaryocytes (precursors to platelets) have been noted in the lungs obtained at autopsy from patients who have died from *status asthmaticus*[23,24], an occurrence that is accompanied with bone marrow karyopoiesis and thrombopoiesis[23]. This is perhaps the result of localized platelet recruitment and activation within lungs[25], since circulating venous platelet numbers have been shown to drop during an early phase response to allergen[26,27]. Circulating platelet aggregates have also been detected in patients with spontaneous asthma attacks[1,3]. Platelets have been observed to accumulate in the microvasculature of lungs from atopic asthmatics[28], and platelets[29,30] and platelet products[31], have been detected in the bronchoalveolar lavage (BAL) fluid, and attached to the lumenal side of the epithelium in patients with late onset airways obstruction. Platelets have also been observed to undergo diapedesis in sections of lung from asthmatic patients[32]. The close association of platelets with eosinophils in BAL fluid[29] has led to the hypothesis that activated platelets may enhance leukocyte migration across endothelium (see below), since it is apparent that platelets carry glycohydrolases in their liposomes, capable of degrading glycoproteins, glycolipids and glycosaminoglycans on cell membranes[33], and thus may facilitate transmigration. Altered platelet function has been shown to be associated with bronchial hyper-responsiveness occurring in nocturnal asthma, where a reduction in the aggregatory response to collagen and PAF was accompanied by lower levels of intraplatelet β-thromboglobulin (β-TG), a platelet specific protein, in a time-dependent phenomenon that occurred concomitantly with a corresponding maximal reduction in peak expiratory flow and a maximal increase in bronchial hyper-responsiveness to methacholine[34].

Raised levels of mediators peculiar to platelets are observed in atopic individuals compared to normal individuals during allergen exposure. The release of platelet specific granular products such as β-TG, and platelet factor 4 (PF4) are increased during symptomatic periods of asthma[35], and are observed in plasma and BAL fluid of atopic subjects[31,36], and platelets become activated after allergen provocation, as measured by thromboxane (TXA_2) production[37]. Other mediators common to platelets have been observed in atopic patients: PAF levels, as measured by the precursor and metabolite lyso-PAF, are significantly increased in patients suffering a late asthmatic response, when compared to the immediate response[38,39]. Nedocromil sodium, a drug used in the treatment of asthma, has been shown to inhibit platelet activation induced by PAF in ex vivo studies[40]. This rise in PAF levels may be related to a deficiency in PAF acetylhydrolase, an enzyme that inactivates PAF, in children studied with moderate or severe asthma[41]. The increased release of 'regulated upon activation, normally T-cell expressed and secreted' (RANTES), CD62P (P-selectin) expression and an increase in intracellular free calcium concentration $[Ca^{2+}]_i$ is observed in platelets from asthmatics after thromboxane stimulation[42,43], and the release of these products has been correlated to a rise in β-TG levels in vivo[43]. The increased presence of these mediators has been shown to be suppressed by oral administration of theophylline[42], which may contribute to the efficacy of theophylline in the treatment of asthmatics.

The role of IgE on platelet function in allergies

Production of antigen-specific IgE in response to allergen provocation is a fundamental hallmark of atopic diseases[44,45]. The cross-linking of IgE by antigen is believed to provide the stimulus for mast cell degranulation in early phase reactions, an event that precipitates a cascade of inflammatory events in response to allergen[24,46,47]. Compelling evidence reveals that platelets are also activated via IgE, perhaps initiating an important pathway to platelet activation, independent of mediator release from other inflammatory cells. Patients allergic to *Dermatophagoides pteronyssinus* have been exposed to synthetic peptides derived from the allergen Der p1, and were shown to have specifically activated platelets. This was a process mediated by IgE, that did not stimulate platelets from healthy subjects or non-Der p1 allergic patients, illustrating the selectivity of how platelets respond to a specific allergen stimulus[48]. Similarly, platelets bearing IgE were found to be activated only via specific antigens in IgE dependent killing of *Schistosoma mansoni*[49]. The involvement of platelets in IgE-mediated responses may well represent inappropriate actions of platelets commonly displayed in IgE-mediated immunity against helminth and protozoan parasitic infections[50,51], processes that can be inhibited by T-cell products[49].

Between 20 and 30% of platelets from normal individuals bind IgE, this binding affinity rising up to 50% from patients with allergies[10,52]. Platelets from atopic individuals are characterized by a much greater IgE content compared to non-atopics, and this correlates to the levels of IgE in serum from atopic patients, the stimulation of whose platelets resulted

Cambridge Books Online © Cambridge University Press, 2009

in the release of 65% of stored IgE by PAF stimulation but not by platelet mediators, thrombin and ADP[53]. IgE appears to be stored in platelet α-granules[53]. This is through a specific directional uptake mechanism under the control of intracellular IgE receptors[54,55]. Platelets contain both the high (10^{-9}M) and low affinity (10^{-7}M) receptors for IgE (FcεRI and FcεRII/CD23, respectively) on the surface membrane[50,52,56,57]. However, it is apparent that only low proportions of platelets express both FcεRI and FcεRII simultaneously[58]. The activation of the high affinity receptor on human platelets with monoclonal IgE resulted in the activation of platelets, as demonstrated by platelet cytotoxicity to *Schistomonia mansoni* larvae[58] and the stimulation of platelets via FcεRI has been shown to induce the release of 5-HT and RANTES, demonstrating that platelets may play an important role in the progression of allergic inflammation via IgE-dependent mechanisms[53,57]. Platelets from allergic patients produce free-radical oxygen species in response to IgE stimulation via specific allergens or antibodies[52]. This process of platelet activation could be inhibited by antibodies specific for the low affinity receptor for IgE (fcεRII/CD23)[58], and thus reveals a negative feedback response of platelets to IgE stimulation, governed by the differential up-regulation of IgE receptors. Platelets from asthmatic patients have also been observed to undergo chemotaxis in response to allergen exposure, perhaps via platelet bound, antigen specific IgE[59]. The process of platelet activation by IgE has been demonstrated to be inhibited by drugs used for the treatment of atopic asthma, such as nedocromil sodium and disodium cromoglycate[60,61], resulting in a decrease in the generation of cytotoxic mediators and the inhibition of oxidative metabolism[60], as well as increasing the survival time of platelets from asthmatic patients and increasing monoamine uptake, a process inhibited by IgE[22,62], whilst the inhibition of eosinophil chemotaxis by cetirizine (an H_1 receptor antagonist used in the treatment of allergies) may be linked to the inhibition of IgE dependent platelet activation[63]. Recent evidence has highlighted the possible stimulation of the collagen receptor (GPVI), via mechanisms similar to IgE receptor activation[56], since it is also a member of the Ig superfamily of proteins being associated with the Fc receptor γ chain which can be activated upon cross-linking of antibodies[64,65]. However, observed aggregation of platelets via IgE cross-linking may be the result of platelets self-adhering to each other via P-selectin, since stimulation up-regulates expression in asthmatic individuals[42], and platelets also contain the counter ligand (see below), because it is known that aggregation, as observed in thrombotic events, occurs via different mechanisms to that which stimulates platelet activation in asthmatic patients[11].

Experimental evidence for the role of platelets in animal models of allergy

Experimental animal models have been utilized to better understand the role played by platelets in allergic inflammation. Research in vivo has emphasized the intimate nature in which platelets cooperate with other inflammatory cells, due to the perception that the pathogenesis of atopic asthma is driven by an acquired inflammatory response to allergen via IgE dependent processes, resulting in eosinophil infiltration to the lung and damaged epithelium. Asthma is characterized clinically by hyper-responsiveness of airway smooth muscle in vivo to various spasmogens and, chronically, an accelerated decline in lung function, accompanied by airway wall obstruction and tissue remodelling, which may be the consequence of a cycle of damage and inappropriate repair mechanisms. Evidence now suggests that platelets are recruited to the lung after exposure to allergen and may therefore participate in these various processes, as well as interacting with other effector cells driving the pathogenesis of asthma.

Platelets were first observed to undergo diapedesis into the extravascular tissue of guinea pig lungs following antigen sensitisation, an effect that could be mimicked by exposure to PAF[66]. In particular, platelets were found in close proximity to areas of bronchial smooth muscle and eosinophil infiltration. The actions of PAF were found to be selective since other platelet agonists such as ADP that are capable of inducing platelet activation within the pulmonary vasculature did not induce the diapedesis of eosinophils, suggesting a cohesive link between the two cell types, which is unrelated to stimuli that cause aggregation[66]. Platelets have also been reported in the BAL fluid of allergic rabbits undergoing late onset airways obstruction following antigen challenge[29]. Recent evidence has suggested that platelets may be selectively recruited to sites of inflammation, being targets for certain chemokines, after reports that the chemokine, stromal cell-derived factor-1 (SDF-1), and macrophage-derived chemokine (MDC) activate platelets[67,68] via their receptors CXCR4 and CCR4 respectively. Intravenous administration of PAF and other platelet agonists into guinea pigs also induced bronchospasm, and was associated with an accumulation of platelets in the lung[69–74]. The depletion of platelets in similar models resulted in the abolition of the acute bronchoconstriction to inhaled allergen[75], and has been shown to protect against anaphylaxis in allergic rabbits[71]. The inhibition of bronchoactive agents released by platelets has also been reported to abrogate the resulting bronchospasm in such models[76–78]. Platelets therefore have the

Cambridge Books Online © Cambridge University Press, 2009

capacity, either solely or by interacting with other cell types, to critically affect certain parameters of asthma (see Fig. 56.2).

Platelet derived mediators involved in asthma

Platelets contain a plethora of inflammatory mediators released during allergic responses: preformed mediators, either stored in dense (ADP, ATP, 5-HT, Ca^{2+}) and α granules, containing mediators synthesized by megakaryocytes (e.g. PF4, PDGF) or sequestered in the circulation (e.g. IgE) or lysosomes, which contain matrix metalloproteinases capable of degrading structural tissue, and also the *de novo* production of arachidonic acid metabolites. 5-HT is a vasoconstrictor and increases vascular permeability during inflammatory events[79]. 5-HT has also been shown to induce fibroblast proliferation and thus may contribute to the phenomenon of airway remodelling[80], since drug induced 5-HT uptake by platelets has been shown to reduce the clinical severity in asthmatic patients[14]. Adenosine, also released from dense granules, has been shown to act as a bronchoconstrictor[81]. Platelets are capable of synthesizing and releasing histamine[82,83], which enhances platelet aggregation[84], and is also released during aggregation in vivo[83]. Human platelets may also induce the release of histamine from mast cells and basophils through IgE dependent mechanisms, as well as through thrombin, PAF, and collagen activation, resulting in bronchoconstriction[85,86]. This process is via the release of platelet derived histamine releasing factor (PDHRF), which is also a powerful eosinophil chemoattractant, causing early and late phase airway obstruction and the induction of airways hyper-responsiveness in an allergic rabbit model[87]. Free radical production by platelets may contribute to tissue damage directly[88]. Platelets that release free radicals do not aggregate, and platelet aggregation itself inhibits free radical production[88]. Platelets contain cationic proteins, which enhance vascular permeability to leukocytes[89]. Cationic proteins are also capable of cleaving the fifth component of complement to produce C5a, a chemoattractant to leukocytes[90]. Cationic proteins have also been observed to contribute to tissue damage directly[91]. Of particular interest is the platelet-specific cationic protein PF4, a mediator shown to induce the upregulation of IgG and IgE receptors on eosinophils[92], and stimulates basophils to release histamine[93]. PF4 also acts as a chemokine towards neutrophils, monocytes, fibroblasts, and eosinophils[94] and can activate eosinophils, thus indirectly contributing to the induction of tissue damage and ensuing hyper-responsiveness associated with eosinophil activation[91,95–100]. PF4 has been shown to augment eosinophil adhesion to plates coated with plasma or recombinant soluble intracellular adhesion molecule-1 (rsICAM-1)[101], perhaps via the up-regulation of adhesion molecule expression (leukocyte functional antigen: LFA-1α and LFA-1β) on eosinophils[101], and thus, may be an important mechanism by which PF4 induces hyper-responsiveness to inhaled methacholine in rats, via eosinophil chemoattraction[102]. PF4 is able to reverse immunosuppression by lymphoma cells in mice[103–105]. This was independent of the ability of PF4 to bind to heparin[106], an agent known for its antiproliferative actions[107,108], raising the possibility that platelets are capable of stimulating T-helper 2 lymphocyte proliferation, a T-cell subset believed to orchestrate the inflammatory reaction to allergens.

Other mediators released from the α-granules of platelets have also been shown to induce leukocyte chemotaxis. The stimulation of human platelets with thrombin or via IgE results in the release of RANTES[53,109,110], a mediator that has been shown to be released in asthma[43]. RANTES was demonstrated to act as a chemoattractant towards monocytes and T-lymphocytes[111], and is a potent chemokine for eosinophils[109,112]. RANTES opens Ca^{2+} activated K^{+} channels in eosinophils[113], and may be a mechanism by which eosinophils become activated via chemokine activation, the outcome of which may include the augmentation of eosinophil oxygen free radical production[114].

Other substances released by platelets in allergic reactions are those of arachidonic acid and phospholipid metabolism, produced *de novo* upon activation by a wide variety of inflammatory stimuli[115], the importance of which is illustrated in aspirin sensitive asthma (see below). TXA_2 is a potent vasoconstrictor and smooth muscle spasmogen[116], and platelets interact with macrophages and eosinophils in a co-operative manner, enhancing TXA_2 synthesis[117]. Other arachidonic acid metabolites include the prostaglandins. $PGF_{2\alpha}$ induces platelet aggregation, and has vasoconstrictor properties, whilst PGE_2 acts as a vasodilator and can induce pain[116]. PGE_2 has recently been shown to act as an immunomodulator, with the ability to prime naïve T-cells into producing high levels of T-helper (Th) 2 cytokine expression, cytokines believed to be important in inducing eosinophil recruitment to allergic airways[118]. This action of PGE_2 may be through the inhibition of IL-12 responses (a cytokine that is pivotal in driving the immune system to a Th1 response)[119]. Hydroxyeicosatetraenoic acid (12-HETE) is produced by a platelet specific enzyme (12-lipoxygenase) and has chemotactic activity for eosinophils[120,121]. 12-HETE is also taken up by neutrophils to produce 12, 20-diHETE, a

Cambridge Books Online © Cambridge University Press, 2009

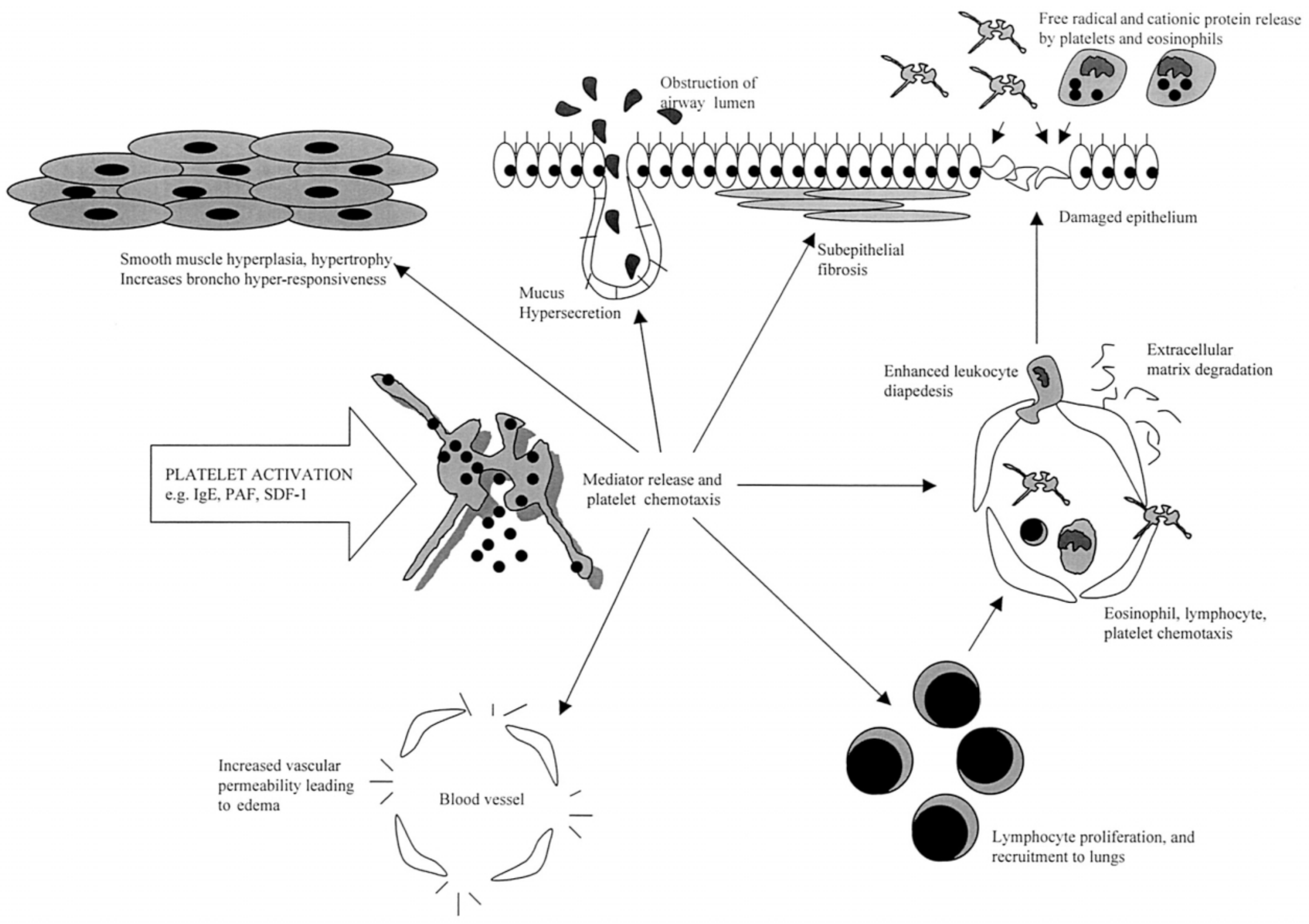


Fig. 56.2. Effects of platelets on cell activation in asthma.

Cambridge Books Online © Cambridge University Press, 2009

chemoattractant that neutrophils are unable to produce in isolation and require activated platelets[121–123]. 12-HETE also stimulates leukocyte 5-lipoxygenase and thus increases leukotriene production[124]. The leukotrienes comprise of cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4) and LTB_4. Cysteinyl leukotrienes are potent inflammatory mediators, inducing bronchospasm, mucus hypersecretion, and increased vascular permeability[125–127], and also increase hyper-responsiveness in asthmatics[128]. LTC_4 and LTD_4 induce eosinophil infiltration into the lungs of guinea-pigs and humans[129,130], whilst LTB_4 acts as a non-specific chemoattractant[131]. Platelets and neutrophils have been shown to cooperate in a synergistic manner with regards to arachidonic acid metabolism. Arachidonic acid from platelets is sequestered by neutrophils with the resulting synthesis of 5-HETE and leukotriene B_4 (LTB_4)[132]. PAF may also enhance LTB_4 production by neutrophils[133]. LTA_4 produced by leukocytes may be converted to LTC_4 by platelets via glutathione-s-transferase via intimate contact between the two cell types[126]. This phenomenon may occur by PMN cells activating platelets via cathepsin-G release, resulting in the expression of P-selectin on platelets[134].

PAF, another phospholipid derived mediator often generated along with arachidonic acid metabolites, is now known to be produced *de novo* by a number of inflammatory cells including platelets via the activation of phospholipase A_2[135]. Many actions of PAF have since been uncovered which are of relevance to allergic inflammation. Guinea pig trachea from animals exposed to aerosolised PAF have been shown to depolarize to a greater extent to histamine than controls, and animals receiving PAF also had eosinophil infiltration into the submucosa[136]. PAF administration has also been shown to induce platelet accumulation into the lungs of baboons and guinea pigs[66,137,138], culminating in the extravasation of platelets into close proximity to areas rich in airway smooth muscle[66], and platelet depletion inhibited PAF induced hyper-responsiveness in rabbits[139]. This phenomenon has been associated with an increase in lung resistance and therefore the changes in lung mechanics may be attributable to platelet activation alone[74], since animals depleted of platelets have a reduction in hyper-responsiveness and eosinophilia[75,140]. The release of substances such as platelet-derived hyper-responsiveness factor (PDHF), after stimulation by PAF may contribute to these effects[141]. PAF is also capable of inducing leukocyte recruitment, perhaps via the ability to induce surface expression of adhesion molecules on eosinophils[101], and the transmigration of eosinophils through artificial membranes has uncovered an important synergistic relationship of PAF and eosinophil stimulating cytokines such as interleukin-5 (IL-5)[142]. Several studies have highlighted the effects of PAF on inflammatory cell product release. The generation of superoxide anion products by formylmethionylleucylphenylalanine (fMLP) stimulated eosinophils is enhanced by preincubation with low concentrations of PAF[143].

Early studies with PAF antagonists in human asthmatics were shown to have no effect on the early or late response to inhaled allergen, or subsequently on airway hyper-responsiveness[144,145]. However, recent evidence suggests other PAF antagonists have activity in asthmatics[146,147], raising the possibility that PAF does play a role in asthma under some circumstances.

Effects of platelets on airway remodelling in asthma

Airway wall thickening, brought about by smooth muscle hypertrophy/hyperplasia, along with subepithelial fibrosis is a common feature in the asthmatic lung, contributing to decline in lung function and increased hyper-responsiveness. Platelets release a number of mitogens that may contribute to bronchial smooth muscle growth and myofibroblast proliferation after continuous platelet activation, since it has been shown that platelets control smooth muscle proliferation in atherosclerosis and restenosis[148]. This is perhaps through the release of chemotactic factors for structural cells, since a platelet-derived factor was found to be chemotactic for rabbit arterial smooth muscle cells in culture[149,150]. Intimal proliferation of smooth muscle, occurring after endothelial injury, has been shown to be preceded by platelet adhesion to the subendothelium[151]. Platelet-derived growth factor (PDGF) has since been shown to affect human, rat, and rabbit tracheal smooth muscle mitogenesis, acting as a potent mitogen for airway smooth muscle cells in culture[152,153], and acts as a potent chemoattractant for fibroblasts[154], as well as monocytes and neutrophils[155]. Recently, PGDF overexpression was observed, along with airway smooth muscle thickening in mice repeatedly exposed to allergen[156]. Transforming growth factor-β (TGFβ), also released by platelets has also been shown to increase smooth muscle cell mitogenesis in culture[157,158], and may contribute to subepithelial fibrosis[159] via its chemotactic properties for fibroblasts and neutrophils[160]. These findings support the hypothesis that platelets are crucial in the induction of chronic airway remodelling in asthmatics due to the granular storage of PDGF and other growth factors released during inflammation.

Cambridge Books Online © Cambridge University Press, 2009

Platelet involvement in leukocyte adherence to endothelium

Platelets, along with eosinophils and monocytes, have been observed attached to the vascular endothelium of asthmatics, an event that was accompanied by extensive inflammatory changes to the airways[28]. A more intimate association between platelets and other inflammatory cells has since been demonstrated by an increase in the number of platelet–leukocyte aggregates during the late asthmatic response[161], an event that correlates with increased platelet activation (see Fig. 56.3). The adhesive events between platelets and leukocytes may be induced by the up-regulation of the adhesion molecule: platelet-selectin (P-selectin), a sialyl Lewis^x tetrasaccharide found on myeloid cells, thus making it possible that leukocytes could adhere to platelets (and endothelium) which carry the P-selectin ligand[162]. P-selectin is stored in the α-granules of platelets and is released to the surface upon activation[163]. Activated platelets have been demonstrated to bind monocytes, PMN cells, eosinophils, basophils and a subpopulation of CD4$^+$ and CD8$^+$ T cells[164,165]. This interaction is dependent on divalent cations, and is completely inhibited by monoclonal antibodies to P-selectin. It has subsequently been shown that circulating activated platelets are capable of reconstituting lymphocyte homing on endothelium in L-selectin deficient mice[166]. P-selectin is also critical in eosinophil recruitment, because it has been demonstrated as the common factor in several selectin combinations for eosinophil attachment to the endothelium[167]. P-selectin deficient mice had no obvious defects in hemostasis, suggesting that P-selectin is only expressed on platelets during inflammatory events[168]. However, in an allergic model of inflammation, mice deficient in P-selectin were shown to have a reduction in eosinophil and lymphocyte accumulation to the lungs, accompanied by a reduction in airways hyper-responsiveness and IL-4 and EPO levels[169]. Furthermore, activated platelets, through P-selectin/sialyl Lewis^x interactions, were shown to be able to induce superoxide anion production in monocytes and neutrophils[170]. Activated platelets are also able to adhere to unstimulated PMN leukocytes, a process involving a P-selectin dependent recognition step and a functional signal that did not require PMN cell activation, thus platelets might directly induce a change in leukocyte activation status[171], via a process dependent on platelet P-selectin recognizing the counter ligand PSGL-1 on PMN cells[172]. Platelet PMN leukocyte interaction in dynamic conditions has also been shown to be dependent on P-selectin interactions with PMN cells resulting in the up-regulation of β_2 integrin CD11b/CD18 on leukocytes[171]. The expression of CD11b/CD18 has subsequently been shown to be most marked in neutrophil populations complexed to platelets[173]. These leukocyte–platelet complexes are found to be the most adhesive cells (respective to their type) in the circulation[174]. P-selectin bound to antigen primed CD4$^+$ T-cells was able to differentially modulate the production of proinflammatory cytokines[175], and thus may facilitate T-cell activation via an interaction with activated platelets. Activated platelets have since been shown to bind to circulating lymphocytes and mediate rolling in high endothelial venules through this interaction with P-selectin and peripheral node addressin, and P-selectin expression on platelets but not endothelial cells was responsible for leukocyte rolling along endothelium[176]. Platelets have also been observed to roll on stimulated endothelium in vivo in a manner similar to leukocyte rolling, being dependent on endothelial, but not on platelet P-selectin[177]. However, mice deficient in P-selectin exhibited virtually no leukocyte rolling in mesenteric venules[168]. Subsequent findings have shown that both human and murine platelets also express PSGL-1, the counter ligand for P-selectin[178], the blocking of which with anti-PSGL-1 antibodies reduced platelet rolling in mesenteric venules[178]. Thus, leukocyte rolling may be enhanced through platelet rosetting around the leukocyte, with subsequent platelet tethering to the endothelial surface, resulting in leukocyte attachment via platelet rosettes. Direct observation of membrane tethering between neutrophils and platelets has been shown under physiological flow conditions[179]. The tethering between spread platelets and free flowing neutrophils was abolished by antibodies to P-selectin and PSGL-1[179]. Platelet rosetting around leukocytes with subsequent attachment and transmigration across endothelium may be a critical process that is further enhanced by the release of platelet lysosomal enzymes degrading glycolipids attached to cell membranes[33], facilitating the movement of leukocytes across the tissue.

Within lysosomes, platelets contain a number of enzymes, termed matrix metalloproteinases[180,181] which are released when platelets become activated[33], and increased levels of which have been observed in the BAL fluid of allergen-challenged subjects[182], and ozone-challenged guinea pigs[183]. These are believed to disrupt the composition and integrity of cell membranes by degrading glycoproteins, glycolipids and glycosaminoglycans. The outcome of this is thought to induce the diapedesis and extravasation of leukocytes, as well as to release membrane-bound growth factors for wound repair[184]. Platelets contain a number of β-hexosaminidases[33], which have been shown to have a mitogenic effect on airway smooth muscle proliferation[185,186]. The implications of

Cambridge Books Online © Cambridge University Press, 2009

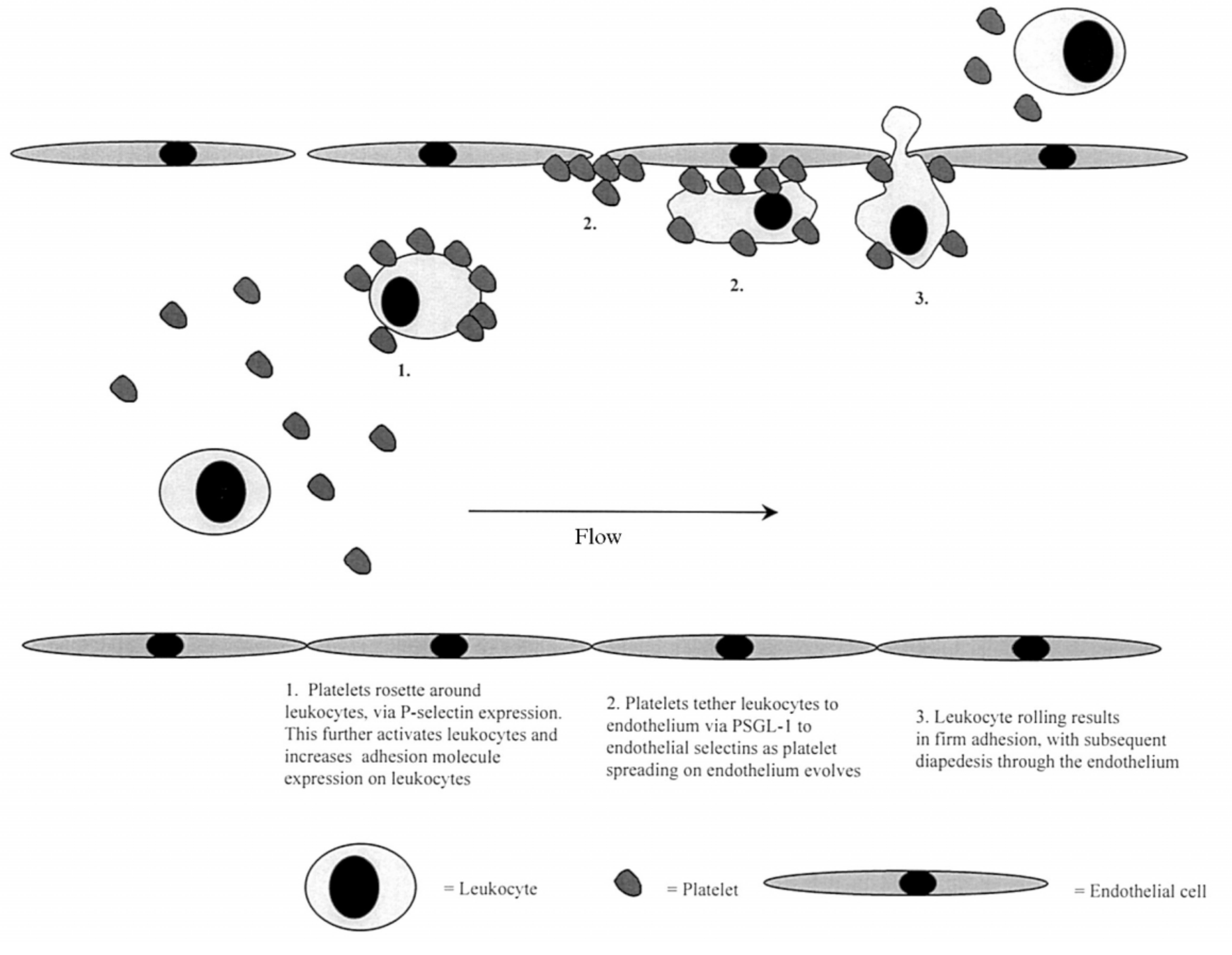


Fig. 56.3. Enhanced leukocyte recruitment via platelet tethering.

Cambridge Books Online © Cambridge University Press, 2009

these actions are profound in the progression of asthma as they may facilitate inflammatory cell extravasation and stimulate tissue remodelling.

The role of platelets in aspirin and NSAID sensitive asthma

Further evidence of platelet involvement in asthma is provided by the growing body of evidence demonstrating that platelets are critically involved in the condition of aspirin induced asthma. Aspirin-induced asthma affects about 10% of adult asthmatics, and is based upon an individual's intolerance to aspirin and other related non-steroidal anti-inflammatory drugs (NSAIDs), due to the pharmacological action of the drug rather than the NSAID inducing an antigen–antibody reaction *per se*. This is demonstrated by the serum of aspirin-sensitive atopic patients being unable to passively sensitise platelets from healthy controls to NSAIDs[187], and the fact that no antibody for NSAIDs has ever been detected.

The phenomenon of aspirin-induced asthma is now thought, in part, to be related to an abnormal response of platelets from such individuals to NSAIDs. Heightened platelet responsiveness to aspirin is recorded in these individuals, this being reduced dramatically after the inhalation of nedocromil sodium[188], a drug that is of clinical benefit in the treatment of aspirin-sensitive asthmatic patients. It has been suggested that the irreversible inhibition of cyclooxygenase (COX) leads to the shunting of arachidonic acid metabolism via the lipooxygenase pathways to increase the production of leukotrienes by platelets[189], especially since leukotriene levels may be enhanced by overproduction of 12-HPETE[124], and the consequences of removing the inhibitory effects of prostaglandins[190]. A decrease in PGE_1, PGE_2, $PGF_{2\alpha}$, and TXB_2 is noted in asthma-sensitive patients[191], whilst higher levels of LTC_4 in nasal secretions and altered levels of LTE_4 have been observed[192]. Lipooxygenase inhibitors are able to prevent the abnormal response of platelets to NSAIDs in vitro[193], and recent clinical studies suggest that such patients respond well clinically to treatment with leukotriene receptor antagonists[194]. However, it is not clear why only a proportion of asthmatic subjects exhibit such an abnormal sensitivity to NSAIDs, and some investigators have reported that aspirin can increase the levels of $PGF_{2\alpha}$, (a bronchoconstrictor) from platelet suspensions obtained from subjects with aspirin-induced asthma, possibly via the displacement of protein-bound $PGF_{2\alpha}$[195], although no changes in β-TG and PGE_2 levels had been observed in this study.

Aspirin-induced asthma has also been linked to the actions of platelet specific glutathione peroxidase activity in such individuals[196]. Glutathione is a tripeptide, playing a key role in detoxification, by reacting with reactive oxygen species, through glutathione peroxidase, an enzyme that has a covalently linked selenium atom at the active site[197]. Serum selenium levels are lower in aspirin-tolerant asthmatics compared to aspirin intolerant asthmatics, and is believed to alter the activity of glutathione peroxidase in this group[196]. Aspirin induces an increase in oxygen-free radical production in aspirin-intolerant asthmatic platelets, although no differences in glutathione peroxidase levels were found between patient groups[198]. The increased production of reactive oxygen species may contribute to the pathogenesis of asthma by causing epithelial damage, leading to an increase in bronchial hyper-responsiveness; since glutathione acts as an antioxidant, and has been recognized as being important in preventing oxidative damage in mice and rats[199,200], and recent evidence has suggested that paracetamol, a drug known to deplete glutathione levels in the lung, increased the clinical severity of asthma, with the incidence of asthma attacks correlating with the frequency of paracetamol usage[201].

Conclusion

A clear dichotomy exists in platelet function, with an inflammatory activity clearly distinguishable from the processes observed during thrombosis and hemostasis. It is now very clear that platelets possess a formidable array of machinery that allows them to play an active role in primary defence mechanisms involving IgE-dependent cytotoxicity to parasites. Allergic insults appear to inappropriately activate these mechanisms by which platelets subsequently contribute to leukocyte infiltration and destruction of the airway architecture leading to a decline in lung function and heightened bronchial responsiveness observed in asthma. There are thus novel possibilities by which the pathogenesis of allergic inflammation can be better understood and perhaps influenced to a greater and better degree than current therapies allow.

References

1 Gresele P, Todisco T, Merante F, Nenci GG. Platelet activation and allergic asthma. *The Lancet* 1982; 306: 549.
2 Gresele P, Grasselli T, Todisco T, Nenci GG. Platelets and asthma. *The Lancet* 1985*;* 8434: 347.

Cambridge Books Online © Cambridge University Press, 2009

3 Gresele P, Ribaldi E, Grasselli T, Todisco T, Nenci GG. Evidence for platelet activation in allergic asthma. *Agents Actions* 1987; Suppl. 21: 119–128.
4 Martin J, Slater D, Trowbridge A. In Schmitz-Schumann M, Menz G, Page C, eds. Platelet production in the lungs. *PAF, platelet and asthma* (Suppl. *Agents and Actions*). Birkhauser-Verlag 1987; 21: 37–57.
5 Storck H, Hoigne R, Koller F. Thrombocytes in allergic reactions. *Int Arch Allergy* 1955; 6: 372–384.
6 Knauer KA, Lichtenstein LM, Adkinson NF, Fish JE. Platelet activation during antigen-induced airway reactions in asthmatic subjects. *N Engl J Med* 1981; 304: 1404–1406.
7 Page CP. In Busse WW, Holgate ST, eds. *Asthma and rhinitis.* This volume.
8 Harker LA, Malpass TW, Branson HE, Hessel II EA, Slichter SJ. Mechanism of abnormal bleeding in patients undergoing cardiopulmonary bypass: acquired transient platelet dysfunction associated with selective a-granule release. *Blood* 1980; 56: 824–834.
9 Pareti FI, Capitanio A, Mannucci L, Ponticelli C, Mannucci PM. Acquired dysfunction due to the circulation of 'exhausted' platelets. *Am J Med* 1980; 69: 235–240.
10 Maccia CA, Gallagher JS, Ataman G, Glueck HI, Brooks SM, Bernstein IL. Platelet thrombopathy in asthmatic patients with elevated immunoglobulin E. *J Allergy Clin Immunol* 1977; 59: 101–108.
11 Palma-Carlos AG, Palma-Carlos ML, Conceicao M, Santos B, Carvalho De Sousa JR. Platelet aggregation in allergic reactions. *Int Arch Allergy Appl Immunol* 1991; 94: 251–253.
12 Ring J, Allen DH, Mathison DA, Spiegelberg HL. *In Vitro* releasability of histamine and serotonin: studies of atopic patients. *J Clin Lab Immunol* 1980; 3: 85–91.
13 Malmgren R, Grubbstrom J, Olsson P, Theorell H, Tornling G, Unge G. Defective serotonin (5-HT) transport mechanism in platelets from patients with endogenous and allergic asthma. *Allergy* 1982; 37: 29–39.
14 Lechin F, Van Der Dijs B, Orozco B, Jara H, Rada I, Lechin ME, Lechin AE. The serotonin uptake-enhancing drug tianeptine suppresses asthmatic symptoms in children: a double-blind, crossover, placebo-controlled study. *J Clin Pharmacol* 1998; 38: 918–925.
15 Yen SS, Morris HG. An imbalance of arachidonic acid metabolism in asthma. *Biochem Biophys Res Commun* 1981; 103: 774–779.
16 Audera C, Rocklin R, Vaillancourt R, Jakubowski A, Deykin D. Altered arachidonic acid metabolism and platelet size in atopic subjects. *Clin Immunol Immunopath* 1988; 46: 352–359.
17 Beer JH, Wuthrich B, Von Felten A. Allergen exposure in acute asthma causes the release of platelet-activating factor (PAF) as demonstrated by the desensitization of platelets to PAF. *Int Arch Allergy Immunol* 1995; 106: 291–296.
18 Block LH, Imhof E, Emmons LR, Roth M, Perruchoud AP. PAF-dependent phoshatidylinositol turnover in platelets: differences between asthmatics and normal individuals. *Respiration* 1990; 57: 372–378.
19 Szczeklik A, Milner PC, Birch J, Watkins J, Martin JF. Prolonged bleeding time, reduced platelet aggregation, altered PAF-acether sensitivity and increased platelet mass are a trait of asthma and hay fever. *Thromb Haemost* 1986; 56: 283–287.
20 Taytard A, Guenard H, Vuillemin L et al. Platelet kinetics in stable atopic asthmatic patients. *Am Rev Resp Dis* 1986; 134: 983–985.
21 Taytard A, Guenard H, Vuillemin L et al. Platelet kinetics in stable asthmatic patients. Effect of corticosteroid therapy. *Am Rev Resp Dis* 1985; 131: A285.
22 Tunon-de-Lara JM, Rio P, Marthan R, Vuillemin L, Ducassou D, Taytard A. The effect of sodium cromoglycate on platelets: an *in vivo* and *in vitro* approach. *J Allergy Clin Immunol* 1992; 89: 994–1000.
23 Slater D, Martin J, Trowbridge A. The platelet in asthma. *Lancet* 1985; 8420: 110.
24 Martin TR, Galli SJ, Katoni IM, Drazen JM. Role of mast cells in anaphylaxis. Evidence for the importance of mast cells in the cardiopulmonary alterations and death induced by anti-IgE in mice. *J Clin Invest* 1989; 83: 1375–1383.
25 Morley J, Sanjar S, Page CP. The platelet in asthma. *Lancet* 1984; 8412: 1142–1144.
26 Maestrelli P, Boschetto P, Zocca E et al. Venous blood platelets decrease during allergen-induced asthmatic reactions. *Clin Exp Allergy* 1990; 20: 367–372.
27 Sullivan PJ, Jaffar ZH, Harbinson PL, Restrick LJ, Costello JF, Page CP. Platelet dynamics following allergen challenge in allergic asthmatics. *Respiration* 2000; 67: 514–517.
28 Beasley R, Roche WR, Roberts JA, Holgate ST. Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am Rev Resp Dis* 1989; 139: 806–817.
29 Metzger WJ, Sjoerdsma K, Richerson HB et al. Platelets in bronchoalveolar lavage from asthmatic patients and allergic rabbits with allergen-induced late phase responses. *Agents Actions* 1987; Suppl. 21: 151–159.
30 Jeffery PK, Wardlaw AJ, Nelson FC, Collins JV, Kay AB. An ultrastructural, quantitative study and correlation with hyperreactivity. *Am Rev Resp Dis* 1989; 140: 1745–1753.
31 Averill FJ, Hubbard WC, Proud D, Gleich GJ, Liu MC. Platelet activation in the lung after antigen challenge in a model of allergic asthma. *Am Rev Resp Dis* 1992; 145: 571–576.
32 Page CP. Platelets. In Holgate ST, Church MK, eds. *Allergy illustrated.* London: Gower Medical Publishing. London. 1993; 8.1–8.8.
33 Ciferri S, Emilliani C, Guglielmini G, Orlacchio A, Nenci GG, Gresele P. Platelets release their lysosomal content in vivo in humans upon activation. *Thromb Haemost* 2000; 83: 157–164.
34 Gresele P, Dottorini M, Selli ML et al. Altered platelet function associated with the bronchial hyperresponsiveness accompanying nocturnal asthma. *J Allergy Clin Immunol* 1993; 91: 894–902.
35 Yasuba H, Chihara J, Kino T, Satake N, Oshima S. Increased releasability of platelet products and reduced heparin induced platelet factor 4 release from endothelial cells in bronchial asthma. *J Lipid Mediat* 1991; 4: 5–21.
36 Yamamoto H, Nagata M, Tabe K et al. The evidence of plate-

Cambridge Books Online © Cambridge University Press, 2009

let activation in bronchial asthma. *J Allergy Clin Immunol* 1993; 91: 79–87.
37 Lupinetti MD, Sheller JR, Catella F, Fitzgerald GA. Thromboxane biosynthesis in allergen-induced bronchospasm. Evidence for platelet activation. *Am Rev Resp Dis* 1989; 140: 932–935.
38 Nakamura T, Morita Y, Kuriyama M, Ishihara K, Ito K, Miyamoto T. Platelet-activating factor in the late asthmatic response. *Int Arch Allergy Appl Immunol* 1987; 82: 57–61.
39 Page CP. A role for platelet-activating factor and platelets in the induction of bronchial hyperreactivity. *Int J Tissue Reactions* 1987; 9: 27–32.
40 Roth M, Soler M, Lefkowitz H et al. Inhibition of receptor-mediated platelet activation by nedocromil sodium. *J Allergy Clin Immunol* 1993; 91: 1217–1225.
41 Miwa M, Miyake T, Yamanaka T et al. Characterisation of serum platelet-activating factor (PAF) acetylhydrolase. *J Clin Investig* 1988; 82: 1983–1991.
42 Moritani C, Ishioka S, Haruta Y, Kambe M, Yamakido M. Activation of platelets in bronchial asthma. *Chest* 1998; 113: 452–458.
43 Chihara J, Yasuba H, Tsuda T et al. Elevation of the plasma level of RANTES during asthma attacks. *J Allergy Clin Immunol* 1997; 100: S52–S55.
44 Burrows B, Martinez FD, Halonen M, Barbee RA, Cline MG. Association of asthma with serum IgE levels and skin-test reactivity to allergens. *N Engl J Med* 1989; 320: 271–277.
45 Hamelmann E, Tadeda K, Oshiba A, Gelfand EW. Role of IgE in the development of allergic inflammation and airway hyperresponsiveness – a murine model. *Allergy* 1999; 54: 297–305.
46 Martin TR, Takeishi T, Katz HR, Austen KF, Drazen JM, Galli SJ. Mast cell activation enhances airway responsiveness to methacholine in the mouse. *J Clin Investig* 1993; 91: 1176–1182.
47 Oshiba A, Hamelmann E, Takeda K. Passive transfer of immediate hypersensitivity and airway hyperresponsiveness by allergen-specific IgE and IgG1 in mice. *J Clin Invest* 1996; 97: 1398–1408.
48 Cardot E, Pestel J, Callebaut I et al. Specific activation of platelets from patients allergic to *Dermatophagoides pteronyssinus* by synthetic peptides derived from the allergen Der p 1. *Int Arch Allergy Immunol* 1992; 98: 127–134.
49 Pancre V, Cesbron JY, Joseph M, Barbier M, Capron A, Auriault C. IgE-dependent killing of *Schistosoma mansoni* schistosomula by human platelets: modulation by T cell products. *Int Archi Allergy Appl Immunol* 1988; 87: 371–375.
50 Joseph M, Auriault C, Capron A, Vorng H, Viens P. A new function for platelets: IgE-dependent killing of schistosomes. *Nature* 1983; 303: 810–812.
51 Momi S, Perito S, Mezzasoma A-M, Bistoni F, Gresele P. Involvement of platelets in experimental mouse trypanosomiasis: evidence of mouse platelet cytoxicity against *Trypanosoma equiperdum*. *Exp Parasitol* 2000; 95: 136–143.
52 Joseph M, Capron A, Ameisen JC et al. The receptor for IgE on blood platelets. *Eur J Immunol* 1986; 16: 306–312.
53 Klouche M, Klinger MHF, Kuhnel W, Wilhelm D. Endocytosis, storage, and release of IgE by human platelets: differences in patients with type 1 allergy and nonatopic subjects. *J Allergy Clin Immunol* 1997; 100: 235–241.
54 Wencel-Drake JD. Plasma membrane GP IIb/IIIa. Evidence for a cycling receptor pool. *Am J Pathol* 1990; 136: 61–70.
55 Wencel-Drake JD, Frelinger AL, Dieter MG, Lam SC. Arg–Gly–Asp-dependent occupancy of GP IIb/IIIa by applaggin: evidence for internalisation and cycling of a platelet integrin. *Blood* 1993: 81: 62–69.
56 Cines DB, Van Der Keyl H, Levinson AL. *In vitro* binding of an IgE protein to human platelets. *J Immunol* 1986; 136: 3433–3440.
57 Hasegawa S, Pawankar R, Suzuki K et al. Functional expression of the high affinity receptor for IgE (Fc∊R1) in human platelets and its' intracellular expression in human megakaryocytes. *Blood* 1999; 93: 2543–2551.
58 Joseph M, Gounni AS, Kusnierz JP et al. Expression and functions of the high-affinity IgE receptor on human platelets and megakaryocyte precursors. *Eur J Immunol* 1997; 27: 2212–2218.
59 Zhang X, Selli ML, Baglioni S. et al. Platelets from asthmatic patients migrate *in vitro* in response to allergen stimulation. *Thromb Haemost* 1993; 69: 1356.
60 Thorel T, Joseph M, Tsicopoulos A, Tonnel AB, Capron A. Inhibition by nedocromil sodium of IgE-mediated activation of human mononuclear phagocytes and platelets in allergy. *Int Arch Allergy Appl Immunol* 1988; 85: 232–237.
61 Tsicopoulos A, Lassalle P, Joseph M, Tonnel B, Dessaint JP, Capron A. Effect of disodium cromoglycate on inflammatory cells bearing the Fc epsilon receptor type II (Fc∊RII). *Int J Immunopharmacol* 1988; 10: 227–236.
62 Tunon-de-Lara JM, Vuillemin L, Rondoyanni F, Rio P, Ducassou D, Taytard A. The inhibitory effect of immunoglobulin E on monoamine uptake in human platelets. *Clin Exp Allergy* 1991; 21: 109–113.
63 De Vos C, Joseph M, Leprevost C. Inhibition of human eosinophil chemotaxis and of the IgE-dependent stimulation of human blood platelets by Cetirizine. *Int Arch Allergy Appl Immunol* 1989; 88: 212–215.
64 Watson S, Berlanga O, Best D, Frampton J. Update on collagen receptor interactions in platelets: is the two-state model still valid? *Platelets* 2000; 11: 252–258.
65 Schulte V, Snell D, Bergmeier W, Zirngibl H, Watson SP, Nieswandt B. Evidence for two distinct epitopes within collagen for activation of murine platelets. *J Biol Chem* 2000; 275: 23998–24002.
66 Lellouch-Tubiana A, Lefort J, Pirotzky E, Vargaftig BB, Pfister A. Ultrastructural evidence for extravascular platelet recruitment in the lung upon intravenous injection of platelet-activating factor (PAF-acether) to guinea-pigs. *Br J Exp Pathol* 1985; 66: 345–355.
67 Kowalska MA, Ratajczak MZ, Majka M. Stromal cell-derived factor-1 and macrophage-derived chemokine: 2 chemokines that activate platelets. *Blood* 2000; 96: 50–57.
68 Abi-Younes S, Sauty A, Mach F, Sukhova GK, Libby P, Luster

Cambridge Books Online © Cambridge University Press, 2009

AD. The stromal cell-derived factor-1 chemokine is a potent platelet agonist highly expressed in atherosclerotic plaques. *Circ Res* 2000; 86: 131–138.
69 Lefort J, Vargaftig BB. Mechanisms of collagen-induced bronchoconstriction and thrombocytopaenia in the guinea-pig. *Br J Pharmacol* 1978; 62: 422P
70 Vargaftig BB, Lefort J. Differential effects of prostacyclin and prostaglandin ϵ1 on bronchoconstriction and thrombocytopenia during collagen and arachidonate infusions and anaphylactic shock in the guinea-pig. *Prostaglandins* 1979; 18: 519–528.
71 Pinckard RN, Halonen M, Palmer JD et al. Intravascular aggregation and pulmonary sequestration of platelets during IgE-induced systemic anaphylaxis in the rabbit: abrogation of lethal anaphylactic shock by platelet depletion. *J Immunol* 1977; 119: 2185–2193.
72 Vargaftig BB, Lefort J, Chignard M, Benveniste J. Platelet-Activating factor induces a platelet-dependent bronchoconstriction unrelated to the formation of prostaglandin derivatives. *Eur J Pharmacol* 1980; 65: 185–192.
73 Dewar A, Archer CB, Paul W, Page CP, Macdonald DM, Morley J. Cutaneous and pulmonary histopathological responses to platelet activating factor (PAF-acether) in the guinea-pig. *J Pathol* 1984; 144: 25–34.
74 Page CP, Paul W, Morley J. Platelets and bronchospasm. *Int Arch Allergy Appl Immunol* 1984; 74: 347–350.
75 Coyle AJ, Page CP, Atkinson L, Flanagan R, Metzger WJ. The requirement for platelets in allergen-induced late asthmatic airway obstruction. *Am Rev Resp Dis* 1990; 142: 587–593.
76 Chignard M, Wal F, Lefort J, Vargaftig BB. Inhibition by sulphinpyrazone of the platelet-dependent bronchoconstriction due to platelet-activation factor (PAF-acether) in the guinea-pig. *Eur J Pharmacol* 1982; 78: 71–79.
77 Vargaftig BB, Lefort J, Wal F, Chignard M, Medeiros MC. Non-steroidal anti-inflammatory drugs if combined with anti-histamine and anti-serotonin agents interfere with the bronchial and platelet effects of 'platelet-activating factor' (PAF-Acether). *Eur J Pharmacol* 1982; 82: 121–130.
78 Chung KF, Aizawa H, Leikauf GD, Ueki IF, Evans TW, Nadel JA. Airway hyperresponsiveness induced by platelet-activating factor: role of thromboxane generation. *J Pharmacol Exp Therap* 1986; 236: 580–584.
79 Majno G, Palade GE. Studies on inflammation. 1. The effect of histamine and serotonin on vascular permeability: an electron microscope study. *J Biophys Biochem Cytol* 1961; 11: 571–605.
80 Boucek RJ, Alvarez TR. 5-hydroxytryptamine: A cyto-specific growth stimulator of cultured fibroblasts. *Science* 1970; 167: 898–899.
81 Holgate ST, Church MK, Polosa R. Adenosine: a positive modulator of airway function in asthma. *Ann NY Acad Sci* 1991; 629: 227–236.
82 Saxena SP, Brandes LJ, Becker AB, Simons KJ, Labella FS, Gerrard JM. Histamine is an intracellular messenger mediating platelet aggregation. *Science* 1989; 243: 1596–1599.
83 Mannaioni PF, Di Bello MG, Raspanti S, Pistelli A, Masini E. Histamine release by human platelets. *Agents Actions* 1993; 38: C203–C205.
84 Mannaioni PF, Di Bello MG, Gambassi F, Mugnai L, Masini E. Platelet histamine: characterisation of the proaggregatory effect of histamine in human platelets. *Int Arch Allergy Appl Immunol* 1992; 99: 394–396.
85 Knauer KA, Kagey-Sobotka A, Adkinson NF, Lichtenstein LM. Platelet augmentation of IgE-dependent histamine release from human basophils and mast cells. *Int Arch Allergy Appl Immunol* 1984; 74: 29–35.
86 Orchard MA, Kagey-Sobotka A, Proud D, Lichtenstein LM. Basophil histamine release induced by a substance from stimulated human platelets. *J Immunol* 1986; 16: 2240–2244.
87 Fisher RH, Henrikson RA, Wirfel-Svet KL, Atkinson L, Metzger WJ. Bronchial challenge with platelet-derived histamine releasing factor (PD-HRF) supernatant induces prolonged changes in dynamic compliance (Cdyn) and hyperreactivity in the allergic asthmatic rabbit model. *J Allergy Clin Immunol* 1990; 85: A472.
88 Joseph M. The generation of free radicals by blood platelets. In Joseph M, ed. *The immunopharmacology of platelets.* Academic Press; 1995: 209–225.
89 Nachman RL, Weksler B, Ferris B. Characterisation of human platelet vascular permeability-enhancing activity. *J Clin Invest* 1972; 51: 549–556.
90 Sasaki M, Paul W, Douglas GJ, Page CP. A comparison of allergen and polycation induced cutaneous responses in the rabbit. *Br J Pharmacol* 2001; 133: 1181–1189.
91 Coyle AJ, Ackerman SJ, Burch R, Proud D, Irwin CG. Human eosinophil major basic protein and synthetic polycations induce airway hyperresponsiveness in vivo dependent on bradykinin generation. *J Clin Invest* 1995; 95: 1735–1740.
92 Chihara J, Fukuda K, Yasuba H. Platelet factor 4 enhances eosinophil IgG and IgE receptors and has eosinophil chemotactic activity. *Am Rev Resp Dis* 1988; 137: A421
93 Brindley LL, Sweet JM, Goetzl EJ. Stimulation of histamine release from human basophil by human platelet factor 4. *J Clin Investig* 1983; 72: 1218–1223.
94 Deuel TF, Senior RM, Chang D, Griffin GL, Heinrikson RL, Kaiser ET. Platelet factor 4 is chemotactic for neutrophils and monocytes. *Proc Natl Acad Sc USA* 1981; 78: 4584–4587.
95 Gleich GJ, Loegering DA, Maldonado JE. Identification of a major basic protein in guinea pig eosinophil granules. *J Exp Med* 1973; 137: 1459–1471.
96 Frigas E, Loegering DA, Gleich GJ. Cytotoxic effects of the guinea pig major basic protein on tracheal epithelium. *Lab Invest* 1980; 42: 35–43.
97 Samoszuk MK, Petersen A, Gidanian F, Rietveld C. Cytophilic and cytotoxic properties of human eosinophil peroxidase plus major basic protein. *Am J Pathol* 1988; 132: 455–460.
98 Motojima S, Frigas E, Loegering DA, Gleich GJ. Toxicity of eosinophil cationic proteins for guinea pig tracheal epithelium in vitro. *Am Rev Resp Dis* 1989; 139: 801– 805.

Cambridge Books Online © Cambridge University Press, 2009

99 Nilsson L, Peterson C, Venge P, Borocviec JW, Thelin S. Eosinophil granule protein in cardiopulmonary bypass with and without heparin coating. *Ann Thorac Surg* 1995; 59: 713–716.
100 Coyle AJ, Perretti F, Manzini S, Irvin CG. Cationic protein-induced sensory nerve activation. *J Clin Investig* 1994; 94: 2301–2306.
101 Hayashi N, Chihara J, Kobayashi Y et al. Effect of platelet-activating factor and platelet factor 4 on eosinophil adhesion. *Int Arch Allergy Immunol* 1994; 104: 57–59.
102 Coyle AJ, Ackerman SJ, Irvin CG. Cationic proteins induce airway hyperresponsiveness dependent on charge interactions. *Am Rev Resp Dis* 1993; 147: 896–900.
103 Katz IR, Hoffman MK, Zucker MB, Bell MK, Thorbecke GJ. A platelet derived immunoregulatory serum factor with T-cell affinity. *J Immunol* 1985; 134: 3199–3203.
104 Katz IR, Hoffman MK, Thorbecke GJ. Lymphoma related immunosuppression in mice is abolished by factors in normal mouse serum. *Federal Proc* 1985; 42: 466.
105 Katz IR, Thorbecke GJ, Bell MK, Yin JZ, Clarke D, Zucker MB. Protease induced immunoregulatory activity of platelet factor 4. *Proc Natl Acad Sci USA* 1986; 83: 3491–3495.
106 Katz IR, Zucker MB, Thorbecke GJ. Platelet factor 4 (PF4) and its c-terminal peptides reverse the suppresser effect of con a-induced blast cells on antibody production *in vitro*. *FASEB J* 1989; 3: A1082
107 Dziarski R. Enhancement of mixed lymphocyte reaction and cytotoxic antitumor responses by heparin. *J Immunol* 1989; 143: 356–365.
108 Gorski A, Wasik M, Nowaczyk M, Korczak-Kowalska G. Immunomodulating activity of heparin. *FASEB J* 1991; 5: 2287–2291.
109 Kameyoshi Y, Doorschner A, Mallet AI, Christopher E, Schroeder JM. Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. *J Exp Med* 1992; 176: 582–591.
110 Kameyoshi Y, Schroder JM, Christopher E, Yamamoto S. Identification of the cytokine RANTES released from platelets as an eosinophil chemotactic factor. *Int Arch Allergy Immunol* 1994; 104: 49–51.
111 Schall TJ, Bacon K, Toy KJ, Goeddel DV. Selective attraction of monocytes and T-lymphocytes of the memory phenotype by cytokine RANTES. *Nature* 1990; 347: 669–671.
112 Rot A, Krieger M, Brunner T, Bischoff SC, Schall TJ, Dahinden CA. RANTES and macrophage inflammatory protein 1α induce the migration and activation of normal human eosinophil granulocytes. *J Exp Med* 1992; 176: 1489–1495.
113 Saito M, Sato R, Hisatome I, Narahashi T. RANTES and platelet-activating factor open Ca^{2+}-activated K^+ channels in eosinophils. *FASEB J* 1996; 10: 792–798.
114 Chihara J, Yamada H, Yamamoto T et al. Priming effect of RANTES on eosinophil oxidative metabolism. *Allergy* 1998; 53: 1178–1182.
115 Vargaftig BB, Lefort J, Wal F, Chignard M. Role of the metabolites of arachidonic in platelet-dependent and independent experimental bronchoconstriction. *Bull Eur Pathophysiol Resp* 1981; 17: 723.
116 Samuelsson B, Goldyne M, Granstrom E, Hamberg M, Hammarstrom S, Malmsten C. Prostaglandin and thromboxanes. *Annu Rev Biochem* 1978; 47: 997–1029.
117 Maghni K, Carrier J, Cloutier S, Sirois P. Cell–cell interactions between platelets, macrophages, eosinophils and natural killer cells in thromboxane A2 biosynthesis. *J Lipid Mediat* 1993; 6: 321–332.
118 Demeure CE, Yang L-P, Desjardins C, Raynauld P, Delespesse G. Prostaglandin E_2 primes naïve T-cells for the production of anti-inflammatory Cytokines. *Eur J Immunol* 1997; 27: 3526–3531.
119 Wu C-Y, Wang K, McDyer JF, Seder RA. Prostaglandin E_2 and dexamethasone inhibit IL-12 receptor expression and IL-12 responsiveness. *J Immunol* 1998; 161: 2723–2730.
120 Goetzl EJ, Woods JM, Gorman RR. Stimulation of human eosinophil and neutrophil polymorphonuclear leukocyte chemotaxis and random migration by 12–L-hydroxy-5,8,10,14-eicosatetraenoic acid. *J Clin Invest* 1977; 59: 179–183.
121 Marcus AJ, Safier LB, Ullman HL et al. 12S,20-dihydroxyicosatetraenoic acid: a new eicosanoid synthesised by neutrophils from 12S-hydroxyicosatetraenoic acid produced by thrombin- or collagen-stimulated platelets. *Proc Natl Acad Sci USA* 1984; 81: 903–907.
122 Marcus AJ, Safier LB, Ullman HL, Broekman MJ, Von Schacky C. Studies on the mechanism of omega-hydroxylation of platelet 12-hydroxeicosatetraenoic acid (12–HETE) by unstimulated neutrophils. *J Clin Invest* 1987; 79: 179–187.
123 Marcus AJ, Safier LB, Ullman HL et al. Platelet–neutrophil interactions. *J Biol Chem* 1988; 263: 2223–2229.
124 Maclouf J, Fruteau De Laclos B, Borgeat P. Stimulation of leukotriene biosynthesis in human blood leukocytes by platelet-derived 12-hydroperoxy-icosatetraenoic acid. *Proc Natl Acad Sci USA* 1982; 79: 6042–6046.
125 Lewis RA, Austen KF, Soberman RJ. Leukotrienes and other products of 5-lipoxygenase pathway: biochemistry and relation to pathobiology in human disease. *N Eng J Med* 1990; 323: 645–655.
126 Maclouf J, Murphy RC. Transcellular metabolism of neutrophil-derived leukotriene A_4 by human platelets: a potential source of leukotriene C_4. *J Biol Chem* 1988; 263: 174–181.
127 Piacentini GL, Kaliner MA. The potential roles of leukotrienes in bronchial asthma. *Am Rev Resp Dis* 1991; 143: S96–S99.
128 Adelroth E, Morris MM, Hargreave FE, O'Byrne PM. Airway responsiveness to leukotrienes C_4 and D_4 and to methacholine in patients with asthma and normal controls. *N Eng J Med* 1986; 315: 480–484.
129 Foster A, Chan CC. Peptide leukotriene involvement in pulmonary eosinophil migration upon antigen challenge in the actively sensitised guinea pig. *Int Arch Allergy Appl Immunol* 1991; 96: 279–284.
130 Diamant Z, Hiltermann JT, Van Rensen EL. The effect of inhaled leukotriene D_4 and methacholine on cell differentials

Cambridge Books Online © Cambridge University Press, 2009

in sputum from patients with asthma. *Am J Resp Crit Care Med* 1997; 155: 1247–1253.
131 Seeds EAM, Kilfeather S, Okiji S, Page CP. Role of lipoxygenase metabolites in PAF and antigen induced bronchial hyperresponsiveness and eosinophil infiltration. *Eur J Pharmacol* 1995; 293: 369–376.
132 Marcus AJ, Broekman MJ, Safier LB. Formation of leukotrienes and other hydroxy acids during platelet–neutrophil interactions *in vitro*. *Biochem Biophys Res Commun* 1982; 109: 130–137.
133 Lin AH, Morton DR, Gorman RR. Acetyl glyceryl ether phosphorylcholine stimulates leukotriene B_4 synthesis in human polymorphonuclear leukocytes. *J Clin Invest* 1982; 70: 1058–1065.
134 Maugeri N, Evangelista V, Celardo A et al. Polymorphonuclear leukocyte–platelet interaction: role of P-selectin in thromboxane B2 and leukotriene C4 co-operative synthesis. *Thromb Haemost* 1994; 72: 450–456.
135 Braquet P, Touqui L, Shen TY, Vargaftig BB. Perspectives in platelet-activating factor research. *Pharmacol Rev* 1987; 39: 97–145.
136 Inoue T, Kannan MS. Platelet-activating factor-induced functional changes in the guinea pig trachea *in vitro*. *Resp Physiol* 1989; 77: 157–171.
137 Robertson DN, Page CP. Effect of platelet agonists on airway reactivity and intrathoracic platelet accumulation. *Br J Pharmacol* 1987; 92: 105–111.
138 Arnoux B, Denjean A, Page CP, Nolibe D, Morley J, Benveniste J. Accumulation of platelets and eosinophils in baboon lung after PAF-acether challenge. *Am Rev Resp Dis* 1988; 137: 855–860.
139 Coyle AJ, Spina D, Page CP. PAF-induced bronchial hyperresponsiveness in the rabbit: contribution of platelets and airway smooth muscle. *Br J Pharmacol* 1990; 101: 31–38.
140 Lellouch-Tubiana A, Lefort J, Simon M, Pfister A, Vargaftig BB. Eosinophil recruitment into guinea pig lungs after PAF-acether and allergen administration. *Am Rev Resp Dis* 1988; 137: 948–954.
141 Sanjar S, Smith D, Kristersson A. Incubation of platelets with PAF produces a factor which causes airway hyperreactivity in guinea-pigs. *Br J Pharmacol* 1989; 96: 75P.
142 Okada S, Kita H, George TJ, Gleich GJ, Leiferman KM. Transmigration of eosinophils through basement membrane components *in vitro*: synergistic effects of platelet-activating factor and eosinophil active cytokines. *Am J Resp Cell Mol Biol* 1997; 16: 455–463.
143 Zoratti EM, Sedgwick JB, Bates ME, Vrtis RF, Geiger K, Busse WW. Platelet-activating factor primes human eosinophil generation of superoxide. *Am J Resp Cell Mol Biol* 1992; 6: 100–106.
144 Kuitert LM, Hui KP, Uthayarkumar S et al. Effect of the platelet-activating factor antagonist UK-74,505 on the early and late response to allergen. *Am Rev Resp Dis* 1993; 147: 82–86.
145 O'Byrne PM. Effect of platelet activating factor antagonist WEB 2086, on allergen-induced asthmatic responses. *Thorax* 1993; 48: 594–598.
146 Hozawa S, Haruta Y, Ishioka S, Yamakido M. Effects of a PAF antagonist, Y-24180, on bronchial hyperresponsiveness in patients with asthma. *Am J Resp Crit Care Med* 1995; 152: 1198–1202.
147 Evans DJ, Barnes PJ, Cluzel M, O'Connor BJ. Effect of a potent platelet-activating factor antagonist SR27417A on allergen induced asthmatic responses. *Am J Resp Crit Care Med* 1997; 156: 11–16.
148 Weber AA, Schror K. The significance of platelet-derived growth factors for proliferation of vascular smooth muscle cells. *Platelets* 1999; 10: 77–96.
149 Ihnatowycz IO, Winocour PD, Moore S. A platelet-derived factor chemotactic for rabbit arterial smooth muscle cells in culture. *Artery* 1981; 9: 316–327.
150 Duel TF, Huang JS. Platelet-derived growth factor: structure, function, and roles in normal and transformed cells. *J Clin Invest* 1984; 74: 669–676.
151 Grotendorst GR, Chang T, Seppa HEJ. Platelet-derived growth factor is a chemoattractant for vascular smooth muscle cells. *J Cell Physiol* 1982; 113: 261–266.
152 Hirst SJ, Barnes PJ, Twort CHC. Quantifying proliferation of cultured human and rabbit airway smooth muscle cells in response to serum and platelet-derived growth factor. *Am J Resp Cell Mol Biol* 1992; 7: 574–581.
153 Brar SS, Kennedy TP, Whorton AR, Murphy TM, Chitano P, Hoidal JR. Requirement for reactive oxygen species in serum-induced and platelet-derived growth factor-induced growth of airway smooth muscle. *J Biol Chem* 1999; 274: 20017–20026.
154 Shure D, Senior RM, Griffin GL, Deuel TF. PDGF AA homodimers are potent chemoattractants for fibroblasts and neutrophils, and for monocytes activated by lymphocytes or cytokines. *Biochem Biophysl Res Commun* 1992; 186: 1510–1514.
155 Tzeng DY, Deuel TF, Huang JS, Boehner RL. Platelet-derived growth factor promotes human peripheral monocyte activation. *Blood* 1985; 66: 179–183.
156 Fukuyama S, Inoue H, Koto H et al. Repeated antigen exposure induces airway smooth muscle thickening and PDGF-B over-expression in sensitised mice. *Am J Resp Crit Care Med* 2000; 161: A741.
157 Kilfeather SA, Okona-Mensah KB, Shittu E, Tagoe S, Costello J, Page C. Stimulation of airway smooth muscle cell mitogenesis by transforming growth factor beta (TGF-β1) and inhibition by heparin and explant-conditioned media. *Eur Resp J* 1995; 8: 547s.
158 Okona-Mensah KB, Shittu E, Page C, Costello J, Kilfeather SA. Inhibition of serum and transforming growth factor beta (TGF-β1)-Induced DNA synthesis in confluent airway smooth muscle by heparin. *Br J Pharmacol* 1998; 125: 599–606.
159 Vignola AM. Transforming growth factor-β expression in mucosal biopsies in asthma and chronic bronchitis. *Am J Resp Crit Care Med* 1997; 156: 591–599.
160 Wahl SM, Hunt DA, Wakefield LM. Transforming growth

Cambridge Books Online © Cambridge University Press, 2009

factor type beta induces monocyte chemotaxis and growth factor production. *Proc Natl Acad Sci USA* 1987; 63: 943–945.
161 Ciferri S, O'Connor BJ, Spina D, Page CP, Gresele P. Platelet activation and platelet-leukocyte aggregates during the late asthmatic response. *Eur Resp J* 2000; 16: 1165, 158s.
162 Moore KL, Stults NL, Diaz S et al. Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. *J Cell Biol* 1992; 118: 445–456.
163 Berman CL, Yeo EL, Wencel-Drake JD, Furie BC, Ginsberg MH, Furie BA. Platelet alpha granule membrane protein that is associated with the plasma membrane after activation. Characterisation and subcellular localisation of platelet activation dependent granule–external membrane protein. *J Clin Invest* 1986; 78: 130–137.
164 Rinder HM, Bonan JL, Rinder CS, Ault KA, Smith BR. Dynamics of leukocyte-platelet Adhesion in Whole Blood. *Blood* 1991; 78: 1730–1737.
165 De Brujine-Admiraal LG, Modderman PW, Von Dem Borne AE, Sonnenberg GK. P-selectin mediates Ca^{2+}-dependent adhesion of activated platelets to many different forms of leukocytes: detection by flow cytometry. *Blood* 1992; 80: 134–142.
166 Diacovo TG, Catalina MD, Siegelman MH, Von Andrian UH. Circulating activated platelets reconstitute lymphocyte homing and immunity in L-selectin -deficient mice. *J Exp Med* 1998; 187: 197–204.
167 Gleich GJ. Mechanisms of eosinophil-associated inflammation. *J Allergy Clin Immunol* 2000; 105: 651–663.
168 Mayadas TN, Johnson RC, Rayburn H, Hynes RO, Wagner DD. Leukocyte rolling and extravasation are severely compromised in P-selectin deficient mice. *Cell* 1993; 74: 544–554.
169 De Sanctis GT, Wolyniec WW, Green FHY et al. Reduction of allergic responses in P-selectin-deficient mice. *J Appl Physiol* 1997; 83: 681–687.
170 Tsuji T, Nagata K, Koche J, Todoroki N, Irimura T. Induction of superoxide anion production from monocytes and neutrophils by activated platelets through the P-selectin siayl Lewis X interaction. *J Leukocyte Biol* 1994; 56: 583–587.
171 Evangelista V, Manarini S, Rotondo S et al. Platelet/polymorphonuclear leukocyte interaction in dynamic conditions: evidence of adhesion cascade and cross talk between P-selectin and the β_2 Integrin CD11b/CD18. *Blood* 1996; 88: 4183–4194.
172 Evangelista V, Manarini S, Sideri R et al. Platelet/polymorphonuclear leukocyte interaction: P-selectin triggers protein – tyrosine phosphorylation dependent CD11b/ CD18 adhesion: role of P-selectin glycoprotein ligand-1 (PSGL-1) as a signalling molecule. *Blood* 1999; 93: 876–885.
173 Peters MJ, Heyderman RS, Hatch DJ, Klein NJ. Investigation of platelet – neutrophil interactions in whole blood by flow cytometry. *J Immunol Methods* 1997; 209: 125–135.
174 Peters MJ, Dixon G, Kotowicz KT, Hatch DJ, Heyderman RS, Klein NJ. Circulating platelet-neutrophil complexes represent a subpopulation of activated neutrophils primed for adhesion, phagocytosis, and intracellular killing. *Br J Haematol* 1999; 106: 391–399.
175 Damle NK, Klussman K, Dietsch MT, Mohagheghpour N, Aruffo A. GMP-140 (P-selectin/CD62) binds to chronically stimulated but not resting $CD4^+$ T lymphocytes and regulates their production of proinflammatory cytokines. *Eur J Immunol* 1992; 22: 1789–1793.
176 Diacovo TG, Roth SJ, Buccola JM, Bainton DF, Springer TA. Neutrophil rolling, arrest and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the β_2-integrin CD11b/CD18. *Blood* 1996; 88: 146–157.
177 Frenette PS, Johnson RC, Hynes RO, Wagner DD. Platelets roll on stimulated endothelium in vivo: an interaction mediated by endothelial P-selectin. *Proc Natl Acad Sci USA* 1995; 92: 7450–7454.
178 Frenette PS, Denis CV, Weiss L et al. P-selectin glycoprotein ligand-1 (PSGL-1) is expressed on platelets and can mediate platelet–endothelial interactions *in vivo. J Exp Med* 2000; 191: 1413–1422.
179 Schmidtke DW, Diamond SL. Direct observation of membrane tethers formed during neutrophil attachment to platelets or P-selectin under physiological flow. J Cell Biol 2000; 149: 719–730.
180 Leoncini G, Balestrero F, Maresca M. Lysosomal enzymes in human platelets. *Cell Biochem Funct* 1985; 3: 121–125.
181 Emiliani C, Martino S, Orlacchio A, Vezza R, Nenci GG, Gresele P. Platelet glycohydrolase activities: characterization and release. *Cell Biochem Funct* 1995; 13: 31–39.
182 Tonnel AB, Joseph PH, Gosset E, Fourmer E, Capron A. Stimulation of alveolar macrophages in asthmatic patients after local provocation test. *Lancet* 1983; 8339: 1406–1408.
183 Lew B, Chodimella V, Murlas CG. Guinea pig ozone induced airway hyperreactivity with increased n-acetyl-beta-D-glucosaminidase activity in bronchoalveolar fluid. *Lung* 1990; 168: 273–283.
184 Vlodavsky I, Eldor A, Haimovitz-Friedman A et al. Expression of heparanase by platelets and circulating cells of the immune system: possible involvement in diapedesis and extravasation. *Invasion Metastasis* 1992; 12: 112–127.
185 Lew B, Rattazzi MC. Mitogenic effect of lysosomal hydrolases on bovine tracheal myocytes in culture. *J Clin Investig* 1991; 88: 1969–1975.
186 Lew B, Dempsey KB, Zhao Y, Muthalif M, Fatima S, Malik KU. β-Hexosaminidase-induced activation of p44/42 mitogen-activated protein kinase is dependent on p21ras and protein kinase c and mediates bovine airway smooth-muscle proliferation. *Am J Resp Cell Mol Biol* 1999; 21: 111–118.
187 Joseph M. In Page CP, ed. *The platelet in health and disease.* Oxford: Blackwell Scientific Publications, 1991.
188 Marquette CH, Joseph M, Tonnel AB et al. The abnormal in vitro response to aspirin of platelets from aspirin-sensitive asthmatics is inhibited after inhalation of nedocromil sodium but not of sodium cromoglycate. *Br J Clin Pharmacol* 1990; 29: 525–531.
189 Szczeklik A. The cyclooxygenase theory of aspirin-induced asthma. *Eur Resp J* 1990; 3: 588–593.

Cambridge Books Online © Cambridge University Press, 2009

190 Kuehl FA, Dougherty HW, Ham EA. Interactions between prostaglandins and leukotrienes. *Biochem Pharmacol* 1984; 33: 1–5.
191 Spector SL, Morris HG, Selner JC. Clinical responses and serum prostaglandin levels in aspirin idiosyncrasy. Effect of aspirin and a new nonsteroidal anti-inflammatory agent. *Chest* 1981; 80: 676–681.
192 Kowalski ML. Aspirin sensitive rhinosinusitis and asthma. *Allergy Proc* 1995; 16: 77–80.
193 Ameisen JC, Capron A, Joseph M et al. Aspirin-sensitive asthma: abnormal platelet response to drugs inducing asthmatic attacks. *Int Arch Allergy Appl Immunol* 1985; 78: 438–448.
194 Barnes NC, Miller CJ. Effect of leukotriene receptor antagonist therapy on the risk of asthma exacerbation in patients with mild to moderate asthma: an integrated analysis of zafirlukast trials. *Thorax* 2000; 55: 478–483.
195 Williams WR, Pawlowicz A, Davies BH. *In Vitro* tests for the diagnosis of aspirin-sensitive asthma. *J Allergy Clin Immunol* 1990; 86: 445–451.
196 Pearson DJ, Suarez-Mendez VJ, Day JP, Millar PF. Selenium status in relation to reduced glutathione peroxidase activity in aspirin sensitive asthma. *Clin Exp Asthma* 1991; 21: 203–208.
197 Epp O, Ladenstein R, Wendel A. The refined structure of the selenoenzyme glutathione peroxidase at 0.2 nm resolution. *Eur J Biochem* 1983; 133: 51–69.
198 Plaza V, Prat J, Rosello J et al. *In vitro* release of arachidonic acid metabolites, glutathione peroxidase, and oxygen-free radicals from platelets of asthmatic patients with and without aspirin intolerance. *Thorax* 1995; 50: 490–496.
199 Jenkinson SG, Black RD, Lawrence RA. Glutathione concentrations in rat lung bronchoalveolar lavage fluid: Effects of hyperoxia. *J Lab Clin Med* 1988; 112: 345–351.
200 Smith LJ, Anderson J, Shamsuddin M et al. Effect of fasting on hyperoxic lung injury in mice. The effect of glutathione. *Am Rev Resp Dis* 1990; 141: 141–149.
201 Shaheen SO, Sterne JAC, Songhurst CE, Burney PGJ. Frequent paracetamol use and asthma in adults. *Thorax* 2000; 55: 266–270.

Cambridge Books Online © Cambridge University Press, 2009

Platelet interactions with other cells related to inflammatory diseases

Patrice E. Poubelle and Pierre Borgeat

From the Centre de Recherche en Rhumatologie et Immunologie, Centre de Recherche du CHUQ and Faculté de Médecine, Université Laval, Quebec, Canada

Introduction

Many inflammatory disorders are clinically associated with high blood platelet counts[1–5]. Unlike in primary thrombocytosis where both arterial and venous complications are present[6,7], these reactive or secondary thrombocytoses are mainly asymptomatic. However, when a secondary thrombocytosis is associated with other risk factors, thromboembolic events can occur in the venous system[7]. Interestingly, thrombocytosis observed in inflammatory diseases is associated with elevated serum levels of inflammatory mediators such as interleukin-6[8,9] or P-selectin[10] as opposed to increased blood thrombopoietin in primary thrombocytosis[11]; in addition, platelets from patients with primary thrombocytosis exhibit a decreased 12-lipoxygenase activity[12]. The clinical impact and the significance of such reactive thrombocytosis, most of the time neglected because it is asymptomatic, remain hypothetical. During chronic inflammatory disorders such as rheumatoid arthritis, thrombocytosis is a hallmark of disease activity[13]. Thrombocytosis has also been associated with a poor prognosis of cervical cancer; this was, however, considered as an indirect indicator and no correlation between platelets and tumour growth was noted[14]. On the other hand, thrombocytosis has been reported in progressive systemic sclerosis[1,15], during which Raynaud's phenomenon is an interesting example of a symptom where platelets are implicated through their activation and interaction with other cells such as endothelial cells. Besides the structural narrowing of digital arteries seen in systemic sclerosis[16], the pathogenesis of Raynaud's phenomenon is, at least in part, related to endothelial injury causing platelet activation (release of vasoconstrictive mediators such as thromboxane A_2, serotonin, ADP) and platelet adhesion to the endothelium[15]. Interactions between platelets and endothelial cells have also been implicated in the pathogenesis of hypertrophic osteoarthropathy where the digital clubbing may be due to the release of fibroblast growth factors by large platelets in distal sites[17–19]. Although platelets and their interactions with other cells are mainly reported to enhance the inflammatory process, one has to keep in mind that platelets are also involved in homeostasis of the vasculature and possibly in the down-regulation of tissue inflammation. Hence, secondary thrombocytosis may be contributing to this control. Indirect evidence of such a role has been reported in in vitro experiments with platelets from healthy subjects which exhibited decreased in vitro production of superoxide anion[20] or LTB_4[21] by ionophore A23187-stimulated neutrophils from uremic or allergic patients, respectively. In the same experiments, platelets from uremic or allergic patients did not show a similar inhibitory activity[20,21]. In vitro experiments also demonstrated that interactions between platelets and endothelial cells, neutrophils or erythrocytes resulted in an active transcellular metabolism of eicosanoids leading to the formation of new bioactive compounds[22–24]. On the other hand, circulating platelets bind to neutrophils and platelet–neutrophil complexes have been found in blood[25]. Unactivated platelets preferentially bind to resting monocytes as compared to neutrophils, at a ratio of three platelets per monocyte[26]. Such cellular complexes may have pathophysiological relevance since, for example, at rest, 25 to 34% of normal blood neutrophils bind platelets and the percentage reaches 70 to 82% after platelet activation[25,27]. Neutrophils and platelets of such cell complexes constitute a subpopulation of activated cells; neutrophils with rosetted platelets have been shown to express higher levels of CD11b (a marker of cell activation) and lower levels of CD62L (L-selectin), have greater capacity to ingest bacteria, and show higher production of superoxide anion than free neutrophils[28]. Cardiopulmonary bypasses, unstable

angina and acute myocardial infarction are clinical conditions where adhesion of platelets to leukocytes is reportedly increased in blood[29–31]. Moreover, low circulating platelet–neutrophil complexes observed in multiorgan failure is associated with a poor prognosis[32]. Depending on the pathophysiological conditions, platelets may regulate inflammation by inducing or by reducing phlogogenic events of the inflammatory reaction. Although in vitro experiments and animal models allow us to explore the regulatory role of platelets in inflammation and the mechanisms involved in such platelet functions, our knowledge of these potentially important homeostatic mechanisms remains fragmentary. The consequence of platelet interactions with other cells in the initiation of an inflammatory (and thrombogenic) process (see below) and the numerous possibilities of promising therapeutic approaches through the pharmacological control of these cell–cell interactions justify the past and present research efforts in this area. We will attempt here to summarize the present knowledge and concepts on the consequences of such interactions between platelets and other cells implicated in inflammation.

Phlogogenic functions of platelets

To date, inflammation, thrombosis and hemostasis are recognized as complex situations with overlapping steps and connecting pathways. Interactions between platelets and inflammatory cells take place at various stages of the inflammatory process; they occur in the circulation and are also observed at extravascular inflammatory sites. The presence of such granulocytes in close contact with platelets at inflammatory sites has been reported as early as 1967[33,34] and has been confirmed later in various conditions such as in adult respiratory distress syndrome[35], in immune complex disease in rabbits[36] and in atherosclerosis. Activated platelets undergo homologous aggregation and adhere to endothelial cells, leukocytes and erythrocytes. Platelets can interact with other cells directly by adhesion to cells or indirectly by releasing endogenous factors. The understanding of such interactions is complicated by the fragmentation of platelets following their activation and the generation of platelet microparticles possessing biological activities such as the acceleration of blood coagulation[37], and the binding to, and activation of, neutrophils[38]. Blood platelet microparticles are increased in pathological conditions such as immune-mediated thrombocytopenia and cardiopulmonary bypass[39–41]. In adult respiratory distress syndrome platelets are activated in vivo and express GMP-140 (P-selectin) and thrombospondin on their surface, while no platelet microparticles are present in blood[42]. Activated as well as non-activated platelets may bind to monocytes, neutrophils, eosinophils and basophils[26,43,44]. Thrombin-activated platelets also bind subpopulations of T-lymphocytes, such as natural killer cells (50% are rosetted with platelets), $CD4^+$ (20%) and $CD8^+$ (30%) lymphocytes [44]. Note that platelets can roll on the endothelium and are actively involved in lymphocyte homing in immune responses in vivo[45–47]. Thus, intact platelets and platelet microparticles bound to leukocytes may actively participate in the inflammatory process from blood to tissues. During the early events of an inflammatory response, activated platelets as well as platelet microparticles exhibit catalytic phospholipid surfaces, which facilitate the prothrombinase reaction[48,49]. The subsequent local formation of thrombin leads to activation not only of platelets but also of endothelial cells, leukocytes and mesenchymal cells[50]. Thrombin is chemotactic for neutrophils and directly stimulates them to release tissue kallikrein[51]; this procoagulant factor activates the expression of IL-8 and IL-6 by mononuclear cells[52], and IL-8 and E-selectin by HUVECs[53]. Thus, experiments addressing the impact of thrombin in platelet interactions with other cells have to be cautiously interpreted. It is also important to stress that the presence of thrombin during inflammation in vivo has been reported to influence local and systemic reactions. For instance, thrombin or activated protein C infusion protects the experimental animals from the lethal effects of LPS[54,55]. Activation of protein C in vivo by thrombin during inflammation leads, in the presence of protein S[56], to the formation of a natural anticoagulant which is further involved in host defense against infection[55]. These observations again point to the complexity of the inflammatory process implicating a highly sophisticated network of mediators and cells interacting to initially promote the development of inflammation, then to control the local response and finally to restore homeostasis.

Interactions of platelets with cells involved in inflammation

Proinflammatory or phlogogenic activities of platelets are efficient when these anuclear blood cells are activated and/or interact with endothelial cells, leukocytes such as neutrophils or monocytes and erythrocytes. The physiologic goal of an inflammatory response is to remove the foreign agent(s) or substance(s) from injured tissue or organ, to promote repair and to restore local equilibrium. The sustained presence of injurious agents in tissues leads to a chronic inflammatory response with decreased func-

Cambridge Books Online © Cambridge University Press, 2009

tions of the targeted tissue(s) or organ(s). The process of removal of injurious agents during inflammation implicates non-resident cells arising locally from blood. Specialized blood cells exit the microvascular compartment through complex events during which cells are activated and undergo cell–cell interactions. Platelets are among these cells and cross-talks between platelets and other cells, in particular endothelial cells, are a very early event in the inflammatory process.

Platelet interactions with endothelial cells during inflammation

Platelet activation is a prerequisite to their interaction with endothelium. Platelet activation can be achieved in vivo as well as in vitro with various agonists which will induce different levels of platelet reactivity. For instance, thrombin or collagen are potent activators of platelets and cause the release of agents contained in various types of granules[57]. Other agonists such as adenosine diphosphate (ADP) or platelet activation following cardiopulmonary bypasses cause the release of α-granule only[58,59]. α-granules contain, among other substances, fibrinogen (further cleaved to fibrin by thrombin), platelet factor 4 (PF4), β-thromboglobulin, transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and thrombospondin, all mediators of inflammation[60]. These α-granules also contain important adhesion receptors, such as P-selectin (or GMP-40, PADGEM, CD-62) and the glycoprotein IIb/IIIa (GP IIb/IIIa is the receptor for fibrinogen) which, upon platelet activation, are expressed at the level of the plasma membrane, allowing cellular interactions (see below). Platelets certainly have not yet revealed all of their secrets and it must be kept in mind that they remain very unstable anucleate cytoplasmic fragments with vestigial *de novo* protein biosynthesis[61]. Platelets activated by ADP, collagen or thrombin express an IL-1 cell-associated material[62], which causes the expression of ICAM-1 (intercellular adhesion molecule-1), IL-6 and GM-CSF by human endothelial cells[63]. Activated platelets are also able to induce the production of IL-8 by endothelial cells via their membrane-associated IL-1 activity[64]. Subsequent to an injury to the endothelium during inflammation, platelets adhere to components of the subendothelium such as collagen or osteopontin and are thereby activated[65,66]. Such activated platelets have been shown to increase the adherence of granulocytes to endothelial cells in vitro and subsequently to amplify granulocyte-mediated ^{51}Cr release from prelabelled endothelial cells in a mechanism implicating serotonin or its derivatives[67]. Moreover, platelets and neutrophils synergize in enhancing the adherence of neutrophils to endothelial cells in models of myocardial reperfusion injury, an effect involving the expression of platelet P-selectin with subsequent neutrophil activation[68,69]. Platelets directly adhere to the endothelium stimulated by lipopolysaccharides (LPS), TNF-α or IL-1[70] through CD11a-CD54 interactions[71]. Platelet depletion efficiently protects mice against systemic and local reactions induced by injection of LPS, indicating that platelets are directly involved in the vascular lesions and tissue damage. In this model, platelets also actively participate in tissue damage through a concomitant activation of neutrophils[71]. Platelet–endothelial cell interactions in vivo were also characterized in rat mesenteric venules by using intravital microscopy; it was shown that the endotoxin-induced rolling of platelets on endothelium was reduced after GP Ibα immunoneutralization[72]. Platelets are also involved in the initiation of inflammatory responses through the local generation of high concentrations of PF-4. This chemokine, which neither induces chemotaxis of neutrophils nor their random migration[73], has been shown to stimulate neutrophil adhesion to unactivated endothelial cells in vitro and secondary granule exocytosis[74]. Another important interaction of platelets on endothelial cells has been recently underlined by using human platelets activated by thrombin, collagen or ADP plus adrenaline and cultured endothelial cells. Preformed CD40 ligand (CD40L, CD154) in platelets is translocated to the cell surface following platelet activation; moreover, activated platelets directly attached to the endothelium in vivo express CD40L[75], and human endothelial cells express CD40 and are activated upon CD40L–CD40 interactions[76]. Coincubation of activated platelets with endothelial cells leads to the endothelial production of IL-8 and MCP-1, as well as the up-regulation of adhesion molecules (E-selectin, VCAM-1, ICAM-1), and these effects are abrogated by an anti-CD40L antibody[75].

Thus, platelet interactions with endothelial cells in inflammation may occur early in the initiation of the inflammatory process, and could lead to the local formation of proinflammatory cytokines and expression of adhesion molecules. These early events triggered by the adhesion of activated platelets to endothelial cells are likely to contribute to the development of inflammation in its initial phase.

Platelet interactions with neutrophils during inflammation

The inflammatory peptide n-formyl-methionyl-leucyl-phenylalanine (fMLP) triggers the accumulation of neutrophils and platelets at the injection site in rabbits. Neutrophil depletion in this model is associated with

Cambridge Books Online © Cambridge University Press, 2009

reduced accumulation of platelets in the inflammatory sites, indicating that neutrophils are required for local platelet accumulation[77]. Similar platelet–neutrophil interactions resulting in platelet extravasation have also been demonstrated in other in vivo inflammatory conditions such as in immune complex nephritis in rats[78–80], myocardial infarction[31,81,82], cardiopulmonary bypass[29,59], and LPS-associated lesions[71,83]. Numerous in vitro studies have attempted to dissect and characterize platelet–neutrophil interactions in experimental conditions mimicking pathophysiological conditions. The stimulatory activity of platelets on neutrophils was suspected as early as 1972 by Nachman et al., who found that platelets promote the migration of leukocytes into thrombi[84]. This chemotactic activity of platelets towards leukocytes was confirmed by Weksler et al.[85] and the chemoattractant was initially identified as platelet factor 4[86], a finding to be taken cautiously in view of recent reports on the properties of highly purified PF4[73,74]. Platelets have also been shown to increase neutrophil aggregation induced by fMLP[87]. Shear-induced microaggregates of neutrophils, which have been demonstrated in whole blood[88], are due to cooperation between platelets and neutrophils[89]. Leukocytes have been shown to respond to conditioned media of activated platelets and migrate to infectious sites formed of invading bacteria and platelet aggregates[90]. Activated human platelets can also stimulate neutrophil phagocytosis[91,92], and enhance the neutrophil production of superoxide anion in response to fMLP, phorbol esters or zymosan[93,94]. Platelet factors responsible, at least in part, for the stimulatory effects were found to be adenine nucleotides such as ATP/ADP[93,95]. However, it has recently been reported that platelets also release macromolecular activators of phagocytosis by neutrophils. Such factors may include among other possible agents transferrin molecules acting by increasing the binding capacity of neutrophil FcγRII[96,97]. Depending on the agonist used to activate neutrophils, platelet products were shown to exhibit divergent effects. Hence, ATP, ADP, AMP and adenosine stimulated O_2^- responses of human neutrophils activated by immune complexes; in contrast, when neutrophils were challenged with fMLP, ATP and ADP increased the responses, whereas AMP and adenosine inhibited the fMLP-induced superoxide anion production[98]. The increase of superoxide anion production induced by platelets during neutrophil–platelet coincubation with ionophore A23187 was also linked to the release of the purine nucleotide ATP by platelets[99]. Neutrophil cytotoxicity and the release of neutrophil lysosome contents are also increased by coincubation with platelets[67,100,101]. Among neutrophil factors affecting platelets, cathepsin G has been shown to be strongly involved in platelet activation[102,103]. This serine protease stored in azurophil granules and released from fMLP-, C5a- or LTB_4-stimulated neutrophils[104–106] can cause, in addition to matrix damage, recruitment and activation of platelets at inflammatory sites through a specific receptor on platelets[107]. NAP-2 (neutrophil-activating peptide-2), a chemotactic activator of neutrophils present in platelet α-granules[108], is formed from the cleavage of NAP-2 precursors, such as CTAP-III (connective tissue-activating peptide III) and β-thromboglobulin, by cathepsin G[109]. Moreover, neutrophil–platelet interactions create a local protected microenvironment favouring the maintenance of an active cathepsin G[110]. Thus, adhesive interactions between platelets and neutrophils generate microenvironments which are likely to favour high local concentrations of mediators protected from neutralizing plasma inhibitors. The inflammatory and thrombotic processes are tightly linked at the very onset of the inflammatory reaction, and factors locally released in association with cell–cell binding through adhesion receptors cooperatively act to transduce regulatory signals. To date, there is convergent evidence that such in vivo platelet–neutrophil interactions are not only relevant to pathophysiological conditions, but are also occurring at rest, participating in the homeostatic equilibrium of tissues and organs.

Platelet interactions with monocytes during inflammation

Interactions between platelets and monocytes, although often similar to those between platelets and neutrophils, present several interesting differences potentially relevant to the regulation of the inflammatory process. Platelets can adhere to monocytes and monocytoid cells such as U937 or THP-1 cells[43,111,112]. Incubation of monocytes with unactivated and thrombin-activated platelets resulted in the rosetting of 87% and 93% of monocytes, respectively[26]. The kinetics of platelet binding to monocytes and neutrophils were different, with a slow binding of unactivated or activated platelets to monocytes over 30–60 minutes compared to a rapid binding of platelets to neutrophils, a process dependent on divalent cations in monocytes, but not in neutrophils[26]. The binding of platelets to monocytes has been demonstrated to implicate thrombospondin and a membrane receptor named glycoprotein IV[111,113]. Activated platelets stimulate monocytes to phagocytoze solid particles[91], to produce superoxide anion[114], to express cytokines such as IL-1β, IL-8 or MCP-1 (monocyte chemotactic protein-1)[31,115]. RANTES ('regulated upon activation normal T cells expressed presumed secreted') is a CC chemokine stored and released by platelets[115]. The RANTES-mediated platelet expression of IL-8 and MCP-1

Cambridge Books Online © Cambridge University Press, 2009

by monocytes during platelet–monocyte interactions requires P-selectin. Direct interactions between platelets and monocytes were shown to be required for the release of MCP-1 and the translocation of NF-κB in monocytes, insofar as supernatants from activated platelets were not able to induce such translocation[115]. Another issue related to platelet and monocyte interactions and the inflammatory process is the expression of tissue factor activity on monocytes[116], a critical event promoting thrombosis during inflammation[117,118]. Blood clotting is initiated by the action of tissue factor, a transmembrane protein of 47 kD acting as a cofactor and produced by endothelial cells, vascular smooth muscle cells and monocytes. Tissue factor binds factors VII and VIIa leading to a molecular complex acting on factors IX and X. Agonists such as LPS, cytokines and immune complexes stimulate the expression of tissue factor by endothelial cells and monocytes; interactions between endothelial cells and lymphocytes[119,120], and between platelets and monocytes[116,121] also induce the generation of tissue factor. Platelet–neutrophil interactions are involved in the enhancement of tissue factor activity in monocytes exposed to LPS[122]. Although in vitro experiments on the effects of platelets on tissue factor expression by monocytes sometimes provided paradoxical results, platelet interactions with monocytes in vivo have been shown to promote tissue factor formation[115,116,123,124]. The stimulatory activity of platelets on monocyte expression of tissue factor is mediated by P-selectin[116]. Moreover, the presence of a circulating pool of tissue factor has recently been shown[125]; in a process that promotes thrombogenesis, this tissue factor is transfered from monocytes to platelets through direct interactions involving CD15 (a leukocyte membrane-bound carbohydrate or sialyl Lewis^x) and tissue factor itself with platelets[126].

Thus, specific events implicating interactions of platelets with monocytes occur during the local thrombogenic process of inflammation.

Platelet interactions with erythrocytes during inflammation

Erythrocytes can also interact with platelets and enhance their ability to produce arachidonic acid and eicosanoids, to release ADP and serotonin and to increase platelet recruitment and aggregation[127,128]. The influence of erythrocytes on platelet reactivity was observed even when using platelet preparations where thromboxane formation was abrogated by aspirin, ADP removed by apyrase or creatine phosphokinase, and protease activity inhibited by a mixture of antiproteases. These data indicate the putative importance of such interactions during the evolution of an inflammatory reaction.

Transcellular metabolism during platelet interactions with other cells

Interactions of platelets with other inflammatory cells have also been suspected at the level of arachidonic acid metabolism since the pioneer studies of Marcus et al[22]. When coincubated, endothelial cells and platelets released greater quantities of prostacyclin (PGI_2) than endothelial cells alone in similar in vitro conditions[22]. Thus, it was concluded that endothelial cells use platelet-derived endoperoxides to generate more PGI_2[129], a phenomenon also observed in vivo at the site of a bleeding time wound[130]. It has also been shown that endothelial cells, through compartmentalized cyclooxygenases, can generate PGH_2 for intracellular production of PGI_2 and transcellular formation of TxB_2 by aspirin-treated platelets[131]. Platelet microparticles have been shown to activate platelets themselves and endothelial cells through transcellular delivery of arachidonic acid leading to TxB_2 and PGI_2 biosynthesis in platelets and endothelial cells, respectively[132]. Such transfer of arachidonic acid during cellular interactions has also been shown to occur from neutrophils to platelets leading to increased formation of TxB_2[133,134]. An increased formation of TxB_2 by platelets was also recorded during interactions between smooth muscle cells and platelets in vitro[135]. Further studies on metabolic interactions resulting in the generation of products not synthesized by single cell types have indicated the pathophysiological relevance of such a transcellular metabolism in different experimental conditions in vitro as well as in vivo. Thus, 12S-hydroperoxy-5,8,10,14(Z,Z,E,Z)-eicosatetraenoic acid (12-HPETE) generated by platelets has been reported to stimulate the production of LTB_4 by human blood neutrophils[136], a phenomenon also observed in pathophysiological states in studies demonstrating the decreased LTB_4 synthesis by neutrophils in the presence of lipoxygenase-deficient platelets from patients with myeloproliferative disorders[137]. It was also demonstrated that neutrophils coincubated with platelets in the presence of ionophore A23187 can transform the platelet product 12S-hydroxy-5,8,10,14(Z,Z,E,Z)-eicosatetraenoic acid (12-HETE) into 5S,12S-dihydroxy-6,8,10,14(E,Z,E,Z)-eicosatetraenoic acid (5S,12S-diHETE)[23,138]. In addition, 12-HETE produced by platelets is metabolized to 12S,20-dihydroxy-5,8,10,14(Z,Z,E,Z)-eicosatetraenoic acid (12,20-diHETE) by unstimulated neutrophils[139]. Amplification of LTB_4 biosynthesis in neutrophils by thrombin-activated platelets was observed using the ionophore but also physiological agonists such as zymosan, fMLP, C5a, or platelet-activating factor (PAF) as stimuli for LTB_4 biosynthesis[140,141]. The mechanisms of the stimulatory effect of

Cambridge Books Online © Cambridge University Press, 2009

platelets on neutrophils implicate the release of arachidonic acid (AA) from platelets[23,141]; however, the platelet-derived AA was shown to be mainly converted by human neutrophils into 5-HETE and Δ6-*trans*-LTB_4 isomers and only marginally transformed into LTB_4[142]. Coincubated platelets and monocytes were also shown to be involved in an active transcellular metabolism of eicosanoids different, however, from that reported during platelet–neutrophil interactions. Monocytes were found to provide unesterified AA, 5-HETE and LTA_4 to platelets. No stimulatory effect of platelet 12-HPETE on 5-lipoxygenase of human blood monocytes was reported, and monocytes did not utilize lipoxygenase products released by platelets[143]. LTA_4 from monocytes was converted by platelets to LTC_4[143], a transcellular metabolism previously reported with neutrophil-derived LTA_4[144]. It is also important to point out that, during platelet–neutrophil interactions, the formation of LTC_4 (and TxB_2) is dependent on the expression of P-selectin by platelets activated by the release of cathepsin G from fMLP-stimulated neutrophils[145]. Moreover, by using reverse transcription PCR techniques it was recently confirmed that platelets contain a LTC_4 synthase[146]. Note that platelet–neutrophil cooperation can lead to increased formation of PAF largely originating from neutrophils, an effect only found after stimulation of platelets[147]. To confirm the in vivo functional significance of platelet interactions with neutrophils during inflammation, Evangelista et al. studied rabbits receiving an intravenous infusion of fMLP[148]. This treatment induced an immediate neutropenia and thrombocytopenia with a concomitant increase of TxB_2, LTB_4 and LTE_4 levels in blood. The chemotactic peptide fMLP is known to activate leukocytes, in particular neutrophils, but not platelets. In fMLP-treated animals, platelets were found aggregated on vessel walls and neutrophils were shown to be activated, especially in capillary lumen of lungs. When rabbits were pretreated with an antiplatelet antiserum, circulating LTB_4 and LTE_4 in fMLP-treated animals were lower than in control rabbits, unlike fMLP-treated rabbits rendered leukopenic where LTB_4 and LTE_4 levels were similar to controls. This interesting report brings invaluable insights into the importance of in vivo interactions of platelets with other cells in the generation of arachidonic acid-derived metabolites involved in acute inflammation.

The transcellular metabolism occurring in platelet-neutrophil interactions also gives rise to other lipoxygenase-derived compounds, the lipoxins. These eicosanoids are generated by the sequential action of two lipoxygenases (LO), either 5–LO and 15–LO or 5–LO and 12–LO on AA[149,150]. Thus, platelets were reported to convert neutrophil-derived (or exogenous) LTA_4 to lipoxins, including the bioactive lipoxin A_4 (LXA_4, 5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid)[151]; lipoxin formation was also shown in ligand-activated platelet and neutrophil coincubations in vitro[152]. Neutrophil–platelet interactions have been shown to occur in inflamed glomeruli during immune complex-mediated glomerulonephritis in rats and mice with subsequent transcellular formation of LXA_4, as assessed by a specific ELISA[80,153].

Mechanisms of interactions between platelets and inflammatory cells

As discussed in the preceding paragraphs, interactions between platelets and other inflammatory cells involve complex mechanisms implicating direct cellular contact and multiple soluble mediators. Cells express on their surface adhesion molecules with binding activity to cognate ligands, also named counter-receptors, which are either expressed on other cells allowing intercellular adhesion, or are soluble. Activated platelets express on their surface several adhesion molecules, which can also participate in the subsequent activation of adherent cells through activation of signalling pathways, such as the P-selectin (or GMP140, PAGDEM, CD62)[154] undergoing phosphorylation under activation[155,156], GP IIb/IIIa (or integrin αIIbβ3)[157], ICAM-2 (intercellular adhesion molecule-2, an Ig-like protein acting as a ligand of β2 integrin)[158], and GPIbα (belonging to the GPIb–IX–V complex)[159], which has recently been implicated in LPS-stimulated interactions of platelets with endothelium[72]. It is important to point out that in the initiation of the inflammatory process, P-selectin is also expressed by endothelial cells implicated in the capture of blood leukocytes. P-selectin has been shown to play a necessary role in platelet interactions with other cells. Platelet adhesion to leukocytes implicates a calcium-dependent mechanism completely abrogated by an anti-P-selectin antibody indicating the importance of P-selectin in such interactions[44,160,161]. The process of rolling and firm adhesion of leukocytes on activated platelets precluding inflammation also involves P-selectin[162–164]. The LPS-induced neutropenia in vivo has been shown to be inhibited by anti-P-selectin antibodies, suggesting that P-selectin is implicated in the early entrapment of leukocytes in tissues such as lungs, kidneys and liver[165]. Using mutant mice lacking P-selectin, Hartwell et al. recently demonstrated the contribution of this receptor in the development of a delayed-type hypersensitivity reaction and a surprising antiinflammatory effect of P-selectin in the immunologically induced glomerulonephritis[47]. Neutrophils and monocytes show increased production of superoxide

Cambridge Books Online © Cambridge University Press, 2009

anion in the presence of activated platelets, a phenomenon inhibited by antibodies to P-selectin[94,114]. P-selectin is also involved in the platelet–leukocyte interaction-dependent formation of TxB_2, LTC_4, and cytokines[145,166]. The counter-receptor for P-selectin has also been the focus of great attention[167]. This ligand, a mucin-like protein named PSGL-1 (P-selectin glycoprotein ligand-1), contains oligosaccharide sequences of the sialyl Lewis^X antigen and fucosylated lactosamine[168–170] providing high selectivity but low affinity for P-selectin. In addition, PSGL-1 binding to P-selectin is dependent on tyrosine sulfation[171]. Blood myeloid cells including polymorpho and mononuclear phagocytes, lymphocytes and dendritic cells express PSGL-1[167]. Binding of P-selectin to PSGL-1 is required in various models of inflammation in animals, i.e. cytokine-induced meningitis[172] and immune complexes-induced lung injury[173]. PSGL-1 has also been shown to be implicated in inflammatory conditions by using PSGL-1 antibodies or soluble PSGL-1 blocking neutrophil infiltration towards inflammatory sites[174,175]. Importantly, PSGL-1 could also be implicated in malignancy since P-selectin was shown to be required in platelet adhesion to neuroblastoma, lung cancer and melanoma[176,177]. Thus, the broad implication of this ligand in pathophysiology supports the intense pharmacological research on P-selectin and its ligand.

The other major mechanism in platelet adhesion and interactions with other cells relies on the integrin system, i.e. the platelet integrin GPIIb/IIIa, and the leukocyte β_2 (CD18) integrins combined with the three different α subunits CD11a (LFA-1), CD11b (Mac-1) or CD11c (GP 150,95). Both platelet GPIIa/IIIb and leukocyte CD11b/CD18 bind fibrinogen through different sequences on fibrinogen chains. Antifibrinogen antibodies decrease the interaction of platelets with leukocytes[178]. Thus, elevated levels of fibrinogen during inflammation contribute to the down-regulation of leukocyte migration at inflammatory sites, insofar as chemoattractants such as PAF (more than LTB_4) promote neutrophil accumulation and adhesion on activated platelets in flow conditions[178]. Endogenous PAF from platelets has also been implicated as a juxtacrine mediator of P-selectin-dependent neutrophil rolling spontaneously leading to a CD18-dependent adhesion[179]. The role of neutrophil-derived PAF has already been demonstrated in neutrophil–platelet interactions triggered by fMLP or LTB_4, a phenomenon blocked by a peptidomimetic of the fibrinogen binding sequence Arg–Gly–Asp–Phe[180]. Moreover, human neutrophils activated in vitro by adhesion to adsorbed fibrinogen undergo induction of IL-1, -8, -6 and TNF-α (as evaluated by RT-PCR, Northern blotting and ELISA techniques); these data were confirmed in vitro using neutrophils from CD18-deficient mice and in vivo in a murine model of peritonitis[181]. Using anti-CD18 antibodies, it has been demonstrated that, during C5a-induced myocardial ischemia, the formation of TxB_2 and sequestration of neutrophils were related to CD18-dependent mechanisms[82], and that in dynamic conditions, such as under high-speed rotatory motion, platelet–neutrophil interactions required β_2 integrins[182]. Platelet aggregation induced by various agonists can be blocked by preincubation with an antibody against the integrin GPIIb/IIIa[183]. This platelet integrin is also involved in platelet–neutrophil interactions since tirofiban, an inhibitor of GPIIb/IIIa given to patients with unstable angina, reduced the number of platelets in blood platelet–neutrophil aggregates[184]. Activated platelets can also bind to the endothelium through interactions of their GPIIb/IIIa with the endothelial cell ICAM-1, $\alpha_v\beta_3$ and GPIbα[185]. Another integrin implicated in platelet interactions with other cells is the integrin $\alpha_v\beta_3$ with a β_3 subunit identical to GPIIIa and an α_v subunit homologous to GPIIb. Although $\alpha_v\beta_3$ is not highly expressed by platelets, it could be involved in platelet adhesion since $\alpha_v\beta_3$ binds fibrinogen, and this binding is inhibited by RGD-containing peptides and an antibody raised against GPIIb/IIIa[186]. Elements other than fibrinogen such as thrombospondin, tenascin, osteopontin and collagens can serve as substrates for the attachment of inflammatory cells. From that point of view, monosodium urate crystals, the causal agent of gouty inflammation, can also act as extracellular substrates for cell attachment, insofar as they have been shown to directly activate platelets through GPIIb/IIIa[187] and neutrophils through CD11b/CD18[188]. This could suggest an amplification process for the local capture of inflammatory cells during gouty inflammation.

It is also important to stress that adhesion molecules are involved not only in cell adhesion but also in intracellular signalling. For example, P-selectin stimulates a very early adhesion of monocytes to activated platelets and endothelial cells while inducing the monocytic generation of inflammatory cytokines such as MCP-1 and TNF-α through enhanced nuclear translocation of NF-κB[166]. Fibrinogen binding to CD11b/CD18 in the monocytoid U937 cells activates the transcription factors NFk-B and AP-1[189]. Studies with human neutrophils incubated on fibrinogen-coated surfaces demonstrated that, under TNF-α stimulation, the adhesion-stimulated neutrophils degranulate through a mechanism implicating Src family kinases[190]. Moreover, platelets adhering to neutrophils stimulate protein–tyrosine phosphorylation in neutrophils through P-selectin/PSGL-1 inducing the activation of CD11b/CD18[191].

Cambridge Books Online © Cambridge University Press, 2009

Inhibitory effects of inflammatory cells on phlogogenic reactivity of platelets

While interactions of platelets with other inflammatory cells result in the activation of the latter, it has also been shown that in contrast inflammatory cells down-regulate platelet reactivity. Thus, human neutrophils have been reported to inhibit platelet aggregation and β-thromboglobulin release in whole blood. The effects of neutrophils were shown to depend, at least in part, on ADPase activity, nitric oxide production and antiaggregatory AA-derived metabolites of neutrophils[192]. Neutrophils have been reported to modulate platelet functions through different mechanisms such as the release of hydrogen peroxide[193], nitric oxide[194] or elastase [195]. As another example, unstimulated neutrophils have been shown to inhibit the release of serotonin from platelets activated by collagen or thrombin, an effect which was amplified by addition of antibodies to P-selectin or by preincubation with lipoxygenase inhibitors. The role of neutrophil ecto-nucleotidase or nitric oxide in these inhibitory effects was ruled out[196]. Endothelial cells can also modulate platelet reactivity through prostacyclin and the subsequent activation of platelet adenylate cyclase, through the generation of nitric oxide or through endothelial ectonucleotidase which rapidly metabolizes platelet-derived ADP[197]. Neutrophils also reduce the formation of TxB_2 by activated platelets in vitro[134]. It has also been reported that activation of neutrophils, but not monocytes, was associated with a decreased expression of PSGL-1 and to an inhibition of neutrophil–platelet adhesion as well as dissociation of pre-existing aggregates[198]. Lymphocytes also appear to have the capacity to inhibit platelet aggregation by a mechanism independent of prostaglandins, nitric oxide or adenosine[199].

Such down-modulation of platelet functions by other inflammatory cells may play an important role in the limitation of the inflammatory process and the restoration of homeostasis.

Antiphlogogenic functions of platelets

If platelets exhibit multiple proinflammatory activities, in particular at the onset of the inflammatory reaction, they have also been reported to down-regulate phlogogenic functions of adjacent cells involved in the process. Ginsburg et al. reported that platelet-conditioned media significantly inhibited the chemotaxis of neutrophils[200]. The production of superoxide anion by neutrophils, a basic function in the control of invading microorganisms, has been shown to be down-modulated by platelets[20,201] or platelet-derived products such as PDGF (platelet-derived growth factor), or P-selectin[20,202–204]. Platelets have also been found to inhibit other functions of human neutrophils such as degranulation and chemotaxis[205], synthesis of LTB_4 and PAF[21,134,206], and apoptosis[207]. The inhibitory effect of platelets on neutrophil apoptosis depends on TGF-β_1 acting through the p38 MAPK pathway[208]. Resting platelets have also been found to decrease the adhesion of neutrophils to endothelial cells, an effect independent of adenosine or P-selectin[209]. Angiogenesis is a critical process for tumour growth and tissue proliferation during the chronic inflammatory reaction; the platelet-derived product PF4 has been shown to inhibit angiogenesis and proliferation of hematopoietic progenitor cells[210,211], an effect related to a decreased cdk2 (cyclin E-cyclin-dependent kinase 2) activity[212].

During experimental glomerulonephritis, the presence of recruited neutrophils and platelets at inflammatory foci is associated with enhanced glomerular arachidonic acid metabolism and increased generation of LTB_4 and TxB_2[79]. However, interactions between platelets and neutrophils in this model lead to the local formation of lipoxin A_4 through transcellular metabolism, a compound with inhibitory effects on neutrophil chemotaxis and neutrophil interactions with endothelial, mesangial and epithelial cells[80]. Experimental glomerulonephritis in P-selectin-deficient mice is associated with increased glomerular neutrophils and more severe proteinuria[153]. The inhibitory roles of LXA_4 generated through platelet–neutrophil interactions was confirmed in these P-selectin deficient mice; the infusion of normal platelets restored the generation of LXA_4 as well as the neutrophil influx to the levels seen in wild-type mice with glomerulonephritis[153,213].

Conclusions

Interactions of platelets or platelet microparticles with other cells involved in the inflammatory process appear to be critical to the initiation and normal course of an acute inflammatory reaction. These complex interactions arise very early in the process through the expression of selectins and integrins by activated platelets allowing interactions with other cell types closely implicated in inflammation and with malignant cells. The data already accumulated on these interactions demonstrated numerous proinflammatory functions of platelets, mainly during the very early phase of the inflammatory process, as well as anti-inflammatory properties later in the course of this

Cambridge Books Online © Cambridge University Press, 2009

inflammatory reaction. Many questions remain, however, unanswered about their impact in host defence and various disease states. Additional studies with either selective antibodies to adhesion molecules, soluble adhesion molecules or synthetic antagonists or with gene (adhesion molecules or their counter-receptors) knockout animals will be necessary to further characterize platelet interactions with other cells, their role in modulating inflammatory cell activity and their pathophysiological significance in acute or chronic inflammatory diseases as well as in infectious disorders. Such studies might provide the necessary proof of concept in support of the development of therapeutic approaches to inflammatory diseases based on the modulation of platelet activity, more specifically of their stimulatory interactions with other inflammatory cells.

Acknowledgements

Supported by CIHR, CAN, TAS grants and Laval University.

References

1 Valentini G, Chianese U, Tirri G, Giordano M. [Thrombocytosis in progressive generalized sclerosis (scleroderma) and in other rheumatic diseases]. *Z Rheumatol* 1978; 37: 233–241.

2 Brezis M, Lafair J. Thrombocytosis in chronic eosinophilic pneumonia. *Chest* 1979; 76: 231–232.

3 Farr M, Scott DL, Constable TJ, Hawker RJ, Hawkins CF, Stuart J. Thrombocytosis of active rheumatoid disease. *Ann Rheum Dis* 1983; 42: 545–549.

4 Baynes RD, Bothwell TH, Flax H et al. Reactive thrombocytosis in pulmonary tuberculosis. *J Clin Pathol* 1987; 40: 676–679.

5 Santhosh-Kumar CR, Yohannan MD, Higgy KE, al-Mashhadani SA. Thrombocytosis in adults: analysis of 777 patients. *J Intern Med* 1991; 229: 493–495.

6 Yohannan MD, Higgy KE, al-Mashhadani SA, Santhosh-Kumar CR. Thrombocytosis. Etiologic analysis of 663 patients. *Clin Pediatr (Phila)* 1994; 33: 340–343.

7 Griesshammer M, Bangerter M, Sauer T, Wennauer R, Bergmann L, Heimpel H. Aetiology and clinical significance of thrombocytosis: analysis of 732 patients with an elevated platelet count. *J Intern Med* 1999; 245: 295–300.

8 Hollen CW, Henthorn J, Koziol JA, Burstein SA. Serum interleukin-6 levels in patients with thrombocytosis. *Leuk Lymphoma* 1992; 8: 235–421.

9 Ertenli I, Haznedaroglu IC, Kiraz S, Celik I, Calguneri M, Kirazhi S. Cytokines affecting megakaryocytopoiesis in rheumatoid arthritis with thrombocytosis. *Rheumatol Int* 1996; 16: 5–8.

10 Ertenli I, Kiraz S, Arici M et al. P-selectin as a circulating molecular marker in rheumatoid arthritis with thrombocytosis. *J Rheumatol* 1998; 25: 1054–1058.

11 Wang JC, Chen C, Novetsky AD, Lichter SM, Ahmed F, Friedberg NM. Blood thrombopoietin levels in clonal thrombocytosis and reactive thrombocytosis. *Am J Med* 1998; 104: 451–455.

12 Schafer AI. Deficiency of platelet lipoxygenase activity in myeloproliferative disorders. *N Engl J Med* 1982; 306: 381–386.

13 Hutchinson RM, Davis P, Jayson MI. Thrombocytosis in rheumatoid arthritis. *Ann Rheum Dis* 1976; 35: 138–142.

14 Hernandez E, Lavine M, Dunton CJ, Gracely E, Parker J. Poor prognosis associated with thrombocytosis in patients with cervical cancer. *Cancer* 1992; 69: 2975–2977.

15 Kahaleh MB, Osborn I, Leroy EC. Elevated levels of circulating platelet aggregates and beta-thromboglobulin in scleroderma. *Ann Intern Med* 1982; 96: 610–613.

16 Rodnan GP, Myerowitz RL, Justh GO. Morphologic changes in the digital arteries of patients with progressive systemic sclerosis (scleroderma) and Raynaud phenomenon. *Medicine (Baltimore)* 1980; 59: 393–408.

17 Dickinson CJ, Martin JF. Megakaryocytes and platelet clumps as the cause of finger clubbing. *Lancet* 1987; 2: 1434–1435.

18 Vazquez-Abad D, Martinez-Lavin M. Macrothrombocytes in the peripheral circulation of patients with cardiogenic hypertrophic osteoarthropathy. *Clin Exp Rheumatol* 1991; 9: 59–62.

19 Matucci-Cerinic M, Martinez-Lavin M, Rojo F, Fonseca C, Kahaleh BM. von Willebrand factor antigen in hypertrophic osteoarthropathy. *J Rheumatol* 1992; 19: 765–767.

20 Carulli G, Barsotti G, Cupisti A et al. Platelet-neutrophil interactions in uremic patients: effects on neutrophil superoxide anion production and chemiluminescence. *Nephron* 1995; 69: 248–252.

21 Hosni R, Chabannes B, Pacheco Y et al. Leukotriene B4 levels from stimulated neutrophils from healthy and allergic subjects: effect of platelets and exogenous arachidonic acid. *Eur J Clin Invest* 1991; 21: 631–637.

22 Marcus AJ, Weksler BB, Jaffe EA, Broekman MJ. Synthesis of prostacyclin from platelet-derived endoperoxides by cultured human endothelial cells. J Clin Invest 1980; 66: 979–986.

23 Marcus AJ, Broekman MJ, Safier LB et al. Formation of leukotrienes and other hydroxy acids during platelet-neutrophil interactions in vitro. *Biochem Biophys Res Commun* 1982; 109: 130–137.

24 McGee JE, Fitzpatrick FA. Erythrocyte-neutrophil interactions: formation of leukotriene B4 by transcellular biosynthesis. *Proc Natl Acad Sci USA* 1986; 83: 1349–1353.

25 Rinder HM, Bonan JL, Rinder CS, Ault KA, Smith BR. Dynamics of leukocyte–platelet adhesion in whole blood. *Blood* 1991; 78: 1730–1737.

26 Rinder HM, Bonan JL, Rinder CS, Ault KA, Smith BR. Activated and unactivated platelet adhesion to monocytes and neutrophils. *Blood* 1991; 78: 1760–1769.

Cambridge Books Online © Cambridge University Press, 2009

27 Peters MJ, Heyderman RS, Hatch DJ, Klein NJ. Investigation of platelet–neutrophil interactions in whole blood by flow cytometry. *J Immunol Methods* 1997; 209: 125–135.
28 Peters MJ, Dixon G, Kotowicz KT, Hatch DJ, Heyderman RS, Klein NJ. Circulating platelet-neutrophil complexes represent a subpopulation of activated neutrophils primed for adhesion, phagocytosis and intracellular killing. *Br J Haematol* 1999; 106: 391–399.
29 Rinder CS, Bonan JL, Rinder HM, Mathew J, Hines R, Smith BR. Cardiopulmonary bypass induces leukocyte-platelet adhesion. *Blood* 1992; 79: 1201–1205.
30 Ott I, Neumann FJ, Gawaz M, Schmitt M, Schomig A. Increased neutrophil-platelet adhesion in patients with unstable angina [see comments]. *Circulation* 1996; 94: 1239–1246.
31 Neumann FJ, Marx N, Gawaz M et al. Induction of cytokine expression in leukocytes by binding of thrombin-stimulated platelets. *Circulation* 1997; 95: 2387–2394.
32 Gawaz M, Dickfeld T, Bogner C, Fateh-Moghadam S, Neumann FJ. Platelet function in septic multiple organ dysfunction syndrome [see comments]. *Intens Care Med* 1997; 23: 379–385.
33 Nakao K, Angrist A, Mao P. Collagen and platelet interaction in experimental vegetative cardiac valvular lesions. *Exp Mol Pathol* 1967; 6: 213–222.
34 Prchal JT, Blakely J. Letter: Granulocyte platelet rosettes. *N Engl J Med* 1973; 289: 1146.
35 Bachofen M, Weibel ER. Structural alterations of lung parenchyma in the adult respiratory distress syndrome. *Clin Chest Med* 1982; 3: 35–56.
36 Henson PM, Cochrane CG. Acute immune complex disease in rabbits. The role of complement and of a leukocyte-dependent release of vasoactive amines from platelets. *J Exp Med* 1971; 133: 554–571.
37 Howard MA, Coghlan M, David R, Pfueller SL. Coagulation activities of plasma microparticles. *Thromb Res* 1988; 50: 145–156.
38 Jy W, Mao WW, Horstman L, Tao J, Ahn YS. Platelet microparticles bind, activate and aggregate neutrophils in vitro. *Blood Cells Mol Dis* 1995; 21: 217–231.
39 Khan I, Zucker-Franklin D, Karpatkin S. Microthrombocytosis and platelet fragmentation associated with idiopathic/autoimmune thrombocytopenic purpura. *Br J Haematol* 1975; 31: 449–460.
40 Zucker-Franklin D, Karpatkin S. Red-cell and platelet fragmentation in idiopathic autoimmune thrombocytopenic purpura. *N Engl J Med* 1977; 297: 517–523.
41 Abrams CS, Ellison N, Budzynski AZ, Shattil SJ. Direct detection of activated platelets and platelet-derived microparticles in humans. *Blood* 1990; 75: 128–138.
42 George JN, Pickett EB, Saucerman S et al. Platelet surface glycoproteins. Studies on resting and activated platelets and platelet membrane microparticles in normal subjects, and observations in patients during adult respiratory distress syndrome and cardiac surgery. *J Clin Invest* 1986; 78: 340–348.
43 Jungi TW, Spycher MO, Nydegger UE, Barandun S. Platelet–leukocyte interaction: selective binding of thrombin-stimulated platelets to human monocytes, polymorphonuclear leukocytes, and related cell lines. *Blood* 1986; 67: 629–636.
44 de Bruijne-Admiraal LG, Modderman PW, Von dem Borne AE, Sonnenberg A. P-selectin mediates Ca^{2+}-dependent adhesion of activated platelets to many different types of leukocytes: detection by flow cytometry. *Blood* 1992; 80: 134–142.
45 Diacovo TG, Puri KD, Warnock RA, Springer TA, von Andrian UH. Platelet-mediated lymphocyte delivery to high endothelial venules. *Science* 1996; 273: 252–255.
46 Diacovo TG, Catalina MD, Siegelman MH, von Andrian UH. Circulating activated platelets reconstitute lymphocyte homing and immunity in L-selectin-deficient mice. *J Exp Med* 1998; 187: 197–204.
47 Hartwell DW, Wagner DD. New discoveries with mice mutant in endothelial and platelet selectins. *Thromb Haemost* 1999; 82: 850–857.
48 Zwaal RF, Comfurius P, van Deenen LL. Membrane asymmetry and blood coagulation. *Nature* 1977; 268: 358–360.
49 Sims PJ, Faioni EM, Wiedmer T, Shattil SJ. Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity. *J Biol Chem* 1988; 263: 18205–18212.
50 Grand RJ, Turnell AS, Grabham PW. Cellular consequences of thrombin-receptor activation. *Biochem J* 1996; 313: 353–368.
51 Cohen WM, Wu HF, Featherstone GL, Jenzano JW, Lundblad RL. Linkage between blood coagulation and inflammation: stimulation of neutrophil tissue kallikrein by thrombin. *Biochem Biophys Res Commun* 1991; 176: 315–320.
52 Johnson K, Choi Y, DeGroot E, Samuels I, Creasey A, Aarden L. Potential mechanisms for a proinflammatory vascular cytokine response to coagulation activation. *J Immunol* 1998; 160: 5130–5135.
53 Kaplanski G, Fabrigoule M, Boulay V et al. Thrombin induces endothelial type II activation in vitro: IL-1 and TNF-alpha-independent IL-8 secretion and E-selectin expression. *J Immunol* 1997; 158: 5435–5441.
54 Taylor FB, Jr, Chang A, Hinshaw LB, Esmon CT, Archer LT, Beller BK. A model for thrombin protection against endotoxin. *Thromb Res* 1984; 36: 177–185.
55 Esmon CT, Taylor FB, Jr, Snow TR. Inflammation and coagulation: linked processes potentially regulated through a common pathway mediated by protein C. *Thromb Haemost* 1991; 66: 160–165.
56 Walker FJ. Regulation of activated protein C by a new protein. A possible function for bovine protein S. *J Biol Chem* 1980; 255: 5521–5524.
57 Holmsen H, Kaplan KL, Dangelmaier CA. Differential energy requirements for platelet responses. A simultaneous study of aggregation, three secretory processes, arachidonate liberation, phosphatidylinositol breakdown and phosphatidate production. *Biochem J* 1982; 208: 9–18.

Cambridge Books Online © Cambridge University Press, 2009

58 Kaplan KL, Broekman MJ, Chernoff A, Lesznik GR, Drillings M. Platelet alpha-granule proteins: studies on release and subcellular localization. *Blood* 1979; 53: 604–618.

59 Rinder C, Fitch J. Amplification of the inflammatory response: adhesion molecules associated with platelet/white cell responses. *J Cardiovasc Pharmacol* 1996; 27: S6–S12.

60 Harrison P, Cramer EM. Platelet alpha-granules. *Blood Rev* 1993; 7: 52–62.

61 Kieffer N, Guichard J, Farcet JP, Vainchenker W, Breton-Gorius J. Biosynthesis of major platelet proteins in human blood platelets. *Eur J Biochem* 1987; 164: 189–195.

62 Hawrylowicz CM, Santoro SA, Platt FM, Unanue ER. Activated platelets express IL-1 activity. *J Immunol* 1989; 143: 4015–4018.

63 Hawrylowicz CM, Howells GL, Feldmann M. Platelet-derived interleukin 1 induces human endothelial adhesion molecule expression and cytokine production. *J Exp Med* 1991; 174: 785–790.

64 Kaplanski G, Porat R, Aiura K, Erban JK, Gelfand JA, Dinarello CA. Activated platelets induce endothelial secretion of interleukin-8 in vitro via an interleukin-1-mediated event. *Blood* 1993; 81: 2492–2495.

65 Sixma JJ, Wester J. The hemostatic plug. Semin Hematol 1977; 14: 265–299.

66 Bennett JS, Chan C, Vilaire G, Mousa SA, DeGrado WF. Agonist-activated alphavbeta3 on platelets and lymphocytes binds to the matrix protein osteopontin. *J Biol Chem* 1997; 272: 8137–8140.

67 Boogaerts MA, Yamada O, Jacob HS, Moldow CF. Enhancement of granulocyte–endothelial cell adherence and granulocyte-induced cytotoxicity by platelet release products. *Proc Natl Acad Sci USA* 1982; 79: 7019–7023.

68 Lefer AM, Campbell B, Scalia R, Lefer DJ. Synergism between platelets and neutrophils in provoking cardiac dysfunction after ischemia and reperfusion: role of selectins. *Circulation* 1998; 98: 1322–1328.

69 Kogaki S, Sawa Y, Sano T et al. Selectin on activated platelets enhances neutrophil endothelial adherence in myocardial reperfusion injury. *Cardiovasc Res* 1999; 43: 968–973.

70 Movat HZ, Burrowes CE, Cybulsky MI, Dinarello CA. Acute inflammation and a Shwartzman-like reaction induced by interleukin-1 and tumor necrosis factor. Synergistic action of the cytokines in the induction of inflammation and microvascular injury [published erratum appears in *Am J Pathol* 1988 Mar; 130: 642]. *Am J Pathol* 1987; 129: 463–476.

71 Piguet PF, Vesin C, Ryser JE, Senaldi G, Grau GE, Tacchini-Cottier F. An effector role for platelets in systemic and local lipopolysaccharide-induced toxicity in mice, mediated by a CD11a- and CD54-dependent interaction with endothelium. *Infect Immun* 1993; 61: 4182–4187.

72 Katayama T, Ikeda Y, Handa M et al. Immunoneutralization of glycoprotein Ibalpha attenuates endotoxin-induced interactions of platelets and leukocytes with rat venular endothelium in vivo. *Circ Res* 2000; 86: 1031–1037.

73 Petersen F, Ludwig A, Flad HD, Brandt E. TNF-alpha renders human neutrophils responsive to platelet factor 4. Comparison of PF-4 and IL-8 reveals different activity profiles of the two chemokines. *J Immunol* 1996; 156: 1954–1962.

74 Petersen F, Bock L, Flad HD, Brandt E. Platelet factor 4-induced neutrophil-endothelial cell interaction: involvement of mechanisms and functional consequences different from those elicited by interleukin-8. *Blood* 1999; 94: 4020–4028.

75 Henn V, Slupsky JR, Grafe M et al. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 1998; 391: 591–594.

76 Yellin MJ, Brett J, Baum D et al. Functional interactions of T cells with endothelial cells: the role of CD40L-CD40-mediated signals. *J Exp Med* 1995; 182: 1857–1864.

77 Issekutz AC, Ripley M, Jackson JR. Role of neutrophils in the deposition of platelets during acute inflammation. *Lab Invest* 1983; 49: 716–724.

78 Johnson RJ, Alpers CE, Pritzl P et al. Platelets mediate neutrophil-dependent immune complex nephritis in the rat. *J Clin Invest* 1988; 82: 1225–1235.

79 Wu X, Pippin J, Lefkowith JB. Platelets and neutrophils are critical to the enhanced glomerular arachidonate metabolism in acute nephrotoxic nephritis in rats. *J Clin Invest* 1993; 91: 766–773.

80 Papayianni A, Serhan CN, Phillips ML, Rennke HG, Brady HR. Transcellular biosynthesis of lipoxin A4 during adhesion of platelets and neutrophils in experimental immune complex glomerulonephritis. *Kidney Int* 1995; 47: 1295–1302.

81 Bednar M, Smith B, Pinto A, Mullane KM. Neutrophil depletion suppresses 111In-labeled platelet accumulation in infarcted myocardium. *J Cardiovasc Pharmacol* 1985; 7: 906–912.

82 Fletcher MP, Stahl GL, Longhurst JC. C5a-induced myocardial ischemia: role for CD18-dependent PMN localization and PMN-platelet interactions. *Am J Physiol* 1993; 265: H1750–H1761.

83 Pearson JM, Schultze AE, Jean PA, Roth RA. Platelet participation in liver injury from gram-negative bacterial lipopolysaccharide in the rat. *Shock* 1995; 4: 178–186.

84 Nachman RL, Weksler B. The platelet as an inflammatory cell. *Ann N Y Acad Sci* 1972; 201: 131–137.

85 Weksler BB, Coupal CE. Platelet-dependent generation of chemotactic activity in serum. *J Exp Med* 1973; 137: 1419–1430.

86 Deuel TF, Senior RM, Chang D, Griffin GL, Heinrikson RL, Kaiser ET. Platelet factor 4 is chemotactic for neutrophils and monocytes. *Proc Natl Acad Sci USA* 1981; 78: 4584–4587.

87 Redl H, Hammerschmidt DE, Schlag G. Augmentation by platelets of granulocyte aggregation in response to chemotaxins: studies utilizing an improved cell preparation technique. *Blood* 1983; 61: 125–131.

88 Jen CJ, McIntire LV. Characteristics of shear-induced aggregation in whole blood. *J Lab Clin Med* 1984; 103: 115–124.

89 Rhee BG, Hall ER, McIntire LV. Platelet modulation of polymorphonuclear leukocyte shear induced aggregation. *Blood* 1986; 67: 240–246.

90 Clawson CC, Rao GH, White JG. Platelet interaction with bac-

Cambridge Books Online © Cambridge University Press, 2009

teria. IV. Stimulation of the release reaction. *Am J Pathol* 1975; 81: 411–420.
91 Sakamoto H, Firkin FC, Chesterman CN. Stimulation of leukocyte phagocytic activity by the platelet release reaction. *Pathology* 1984; 16: 126–130.
92 Sakamoto H, Ooshima A. Activation of neutrophil phagocytosis of complement coated and IgG coated sheep erythrocytes by platelet release products. *Br J Haematol* 1985; 60: 173–181.
93 Ward PA, Cunningham TW, McCulloch KK, Phan SH, Powell J, Johnson KJ. Platelet enhancement of O^{2-}. responses in stimulated human neutrophils. Identification of platelet factor as adenine nucleotide. *Lab Invest* 1988; 58: 37–47.
94 Nagata K, Tsuji T, Todoroki N et al. Activated platelets induce superoxide anion release by monocytes and neutrophils through P-selectin (CD62). *J Immunol* 1993; 151: 3267–3273.
95 Sakamoto H, Firkin F. Characterization of leucocyte phagocytic stimulatory material released by activated human platelets. *Br J Haematol* 1984; 57: 49–60.
96 Sakamoto N, Sakamoto H, Tanaka S, Oryu M, Ogawa Y. Effects of platelet release products on neutrophilic Fc gamma receptors. *J Leukoc Biol* 1998; 64: 631–635.
97 Sakamoto H, Ueno M, Yanghong W et al. Glycoprotein Ibalpha-bound thrombin functions as a serine protease to produce macromolecular activators of phagocytosis from platelets. *Biochem Biophys Res Commun* 2000; 270: 377–382.
98 Ward PA, Cunningham TW, McCulloch KK, Johnson KJ. Regulatory effects of adenosine and adenine nucleotides on oxygen radical responses of neutrophils. *Lab Invest* 1988; 58: 438–447.
99 Colli S, Eligini S, Lalli M, Tremoli E. Platelet–neutrophil interaction and superoxide anion generation: involvement of purine nucleotides. *Free Radic Biol Med* 1996; 20: 271–278.
100 Boogaerts MA, Vercelotti G, Roelant C, Malbrain S, Verwilghen RL, Jacob HS. Platelets augment granulocyte aggregation and cytotoxicity: uncovering of their effects by improved cell separation techniques using Percoll gradients. *Scand J Haematol* 1986; 37: 229–236.
101 Del Maschio A, Corvazier E, Maillet F, Kazatchkine MD, Maclouf J. Platelet-dependent induction and amplification of polymorphonuclear leucocyte lysosomal enzyme release. *Br J Haematol* 1989; 72: 329–335.
102 Chignard M, Selak MA, Smith JB. Direct evidence for the existence of a neutrophil-derived platelet activator (neutrophilin). *Proc Natl Acad Sci USA* 1986; 83: 8609–8613.
103 Selak MA, Chignard M, Smith JB. Cathepsin G is a strong platelet agonist released by neutrophils. *Biochem J* 1988; 251: 293–299.
104 Ferrer-Lopez P, Renesto P, Schattner M, Bassot S, Laurent P, Chignard M. Activation of human platelets by C5a-stimulated neutrophils: a role for cathepsin G. *Am J Physiol* 1990; 258: C1100–C1107.
105 Renesto P, Ferrer-Lopez P, Chignard M. Interference of recombinant eglin C, a proteinase inhibitor extracted from leeches, with neutrophil-mediated platelet activation. *Lab Invest* 1990; 62: 409–416.
106 Del Maschio A, Evangelista V, Rajtar G, Chen ZM, Cerletti C, De Gaetano G. Platelet activation by polymorphonuclear leukocytes exposed to chemotactic agents. *Am J Physiol* 1990; 258: H870–H879.
107 Selak MA, Smith JB. Cathepsin G binding to human platelets. Evidence for a specific receptor. *Biochem J* 1990; 266: 55–62.
108 Walz A, Dewald B, von Tscharner V, Baggiolini M. Effects of the neutrophil-activating peptide NAP-2, platelet basic protein, connective tissue-activating peptide III and platelet factor 4 on human neutrophils. *J Exp Med* 1989; 170: 1745–1750.
109 Cohen AB, Stevens MD, Miller EJ, Atkinson MA, Mullenbach G. Generation of the neutrophil-activating peptide-2 by cathepsin G and cathepsin G-treated human platelets. *Am J Physiol* 1992; 263: L249–L256.
110 Evangelista V, Piccardoni P, White JG, de Gaetano G, Cerletti C. Cathepsin G-dependent platelet stimulation by activated polymorphonuclear leukocytes and its inhibition by antiproteinases: role of P-selectin-mediated cell-cell adhesion. *Blood* 1993; 81: 2947–2957.
111 Silverstein RL, Asch AS, Nachman RL. Glycoprotein IV mediates thrombospondin-dependent platelet-monocyte and platelet-U937 cell adhesion. *J Clin Invest* 1989; 84: 546–552.
112 Theilmeier G, Lenaerts T, Remacle C, Collen D, Vermylen J, Hoylaerts MF. Circulating activated platelets assist THP-1 monocytoid/endothelial cell interaction under shear stress. *Blood* 1999; 94: 2725–2734.
113 Silverstein RL, Nachman RL. Thrombospondin binds to monocytes-macrophages and mediates platelet- monocyte adhesion. *J Clin Invest* 1987; 79: 867–874.
114 Tsuji T, Nagata K, Koike J, Todoroki N, Irimura T. Induction of superoxide anion production from monocytes and neutrophils by activated platelets through the P-selectin-sialyl Lewis X interaction. *J Leukoc Biol* 1994; 56: 583–587.
115 Weyrich AS, Elstad MR, McEver RP et al. Activated platelets signal chemokine synthesis by human monocytes. *J Clin Invest* 1996; 97: 1525–1534.
116 Celi A, Pellegrini G, Lorenzet R et al. P-selectin induces the expression of tissue factor on monocytes. *Proc Natl Acad Sci USA* 1994; 91: 8767–8771.
117 Niemetz J, Marcus AJ. The stimulatory effect of platelets and platelet membranes on the procoagulant activity of leukocytes. *J Clin Invest* 1974; 54: 1437–1443.
118 Warr TA, Rao LV, Rapaport SI. Disseminated intravascular coagulation in rabbits induced by administration of endotoxin or tissue factor: effect of anti-tissue factor antibodies and measurement of plasma extrinsic pathway inhibitor activity. *Blood* 1990; 75: 1481–1489.
119 Lyberg T, Galdal KS, Evensen SA, Prydz H. Cellular cooperation in endothelial cell thromboplastin synthesis. *Br J Haematol* 1983; 53: 85–95.
120 Schmid E, Muller TH, Budzinski RM, Pfizenmaier K, Binder K. Lymphocyte adhesion to human endothelial cells induces tissue factor expression via a juxtacrine pathway. *Thromb Haemost* 1995; 73: 421–428.
121 Lorenzet R, Niemetz J, Marcus AJ, Broekman MJ. Enhancement

Cambridge Books Online © Cambridge University Press, 2009

of mononuclear procoagulant activity by platelet 12-hydroxyeicosatetraenoic acid. *J Clin Invest* 1986; 78: 418–423.
122 Halvorsen H, Olsen JO, Osterud B. Granulocytes enhance LPS-induced tissue factor activity in monocytes via an interaction with platelets. *J Leukoc Biol* 1993; 54: 275–282.
123 Osterud B, Bjorklid E. The production and availability of tissue thromboplastin in cellular populations of whole blood exposed to various concentrations of endotoxin. An assay for detection of endotoxin. *Scand J Haematol* 1982; 29: 175–184.
124 Bauer KA, Kass BL, ten Cate H, Hawiger JJ, Rosenberg RD. Factor IX is activated in vivo by the tissue factor mechanism. *Blood* 1990; 76: 731–736.
125 Giesen PL, Rauch U, Bohrmann B et al. Blood-borne tissue factor: another view of thrombosis. *Proc Natl Acad Sci USA* 1999; 96: 2311–2315.
126 Rauch U, Bonderman D, Bohrmann B et al. Transfer of tissue factor from leukocytes to platelets is mediated by CD15 and tissue factor. *Blood* 2000; 96: 170–175.
127 Santos MT, Valles J, Marcus AJ et al. Enhancement of platelet reactivity and modulation of eicosanoid production by intact erythrocytes. A new approach to platelet activation and recruitment. *J Clin Invest* 1991; 87: 571–580.
128 Valles J, Santos MT, Aznar J et al. Erythrocytes metabolically enhance collagen-induced platelet responsiveness via increased thromboxane production, adenosine diphosphate release, and recruitment. *Blood* 1991; 78: 154–162.
129 Schafer AI, Crawford DD, Gimbrone MA, Jr. Unidirectional transfer of prostaglandin endoperoxides between platelets and endothelial cells. *J Clin Invest* 1984; 73: 1105–1112.
130 Nowak J, FitzGerald GA. Redirection of prostaglandin endoperoxide metabolism at the platelet-vascular interface in man. *J Clin Invest* 1989; 83: 380–385.
131 Karim S, Habib A, Levy-Toledano S, Maclouf J. Cyclooxygenase-1 and -2 of endothelial cells utilize exogenous or endogenous arachidonic acid for transcellular production of thromboxane. *J Biol Chem* 1996; 271: 12042–12048.
132 Barry OP, Pratico D, Lawson JA, FitzGerald GA. Transcellular activation of platelets and endothelial cells by bioactive lipids in platelet microparticles. *J Clin Invest* 1997; 99: 2118–2127.
133 Maugeri N, Evangelista V, Piccardoni P et al. Transcellular metabolism of arachidonic acid: increased platelet thromboxane generation in the presence of activated polymorphonuclear leukocytes. *Blood* 1992; 80: 447–451.
134 Hernandez R, Alemany M, Bozzo J, Ordinas A, Bastida E. Influence of co-incubation and cell number of platelets and polymorphonuclear leukocytes in cellular inhibition and activation phenomena. *Thromb Res* 1994; 74: 255–263.
135 Zou M, Anges C. Cell–cell interaction between platelets and IL-1 beta-stimulated vascular smooth muscle cells in synthesis of thromboxane A2. *Prostaglandins Leukot Essent Fatty Acids* 1997; 56: 85–91.
136 Maclouf J, de Laclos BF, Borgeat P. Stimulation of leukotriene biosynthesis in human blood leukocytes by platelet-derived 12-hydroperoxy-icosatetraenoic acid. *Proc Natl Acad Sci USA* 1982; 79: 6042–6046.
137 Kanaji K, Okuma M, Uchino H. Deficient induction of leukotriene synthesis in human neutrophils by lipoxygenase-deficient platelets. *Blood* 1986; 67: 903–908.
138 Borgeat P, Fruteau de Laclos B, Picard S, Drapeau J, Vallerand P, Corey EJ. Studies on the mechanism of formation of the 5S, 12S-dihydroxy-6,8,10,14(E,Z,E,Z)-icosatetraenoic acid in leukocytes. *Prostaglandins* 1982; 23: 713–724.
139 Marcus AJ, Safier LB, Ullman HL et al. 12S,20-dihydroxyicosatetraenoic acid: a new icosanoid synthesized by neutrophils from 12S-hydroxyicosatetraenoic acid produced by thrombin- or collagen-stimulated platelets. *Proc Natl Acad Sci USA* 1984; 81: 903–907.
140 Del Maschio A, Maclouf J, Corvazier E, Grange MJ, Borgeat P. Activated platelets stimulate human neutrophils functions. *Nouv Rev Fr Hematol* 1985; 27: 275–278.
141 Palmantier R, Borgeat P. Transcellular metabolism of arachidonic acid in platelets and polymorphonuclear leukocytes activated by physiological agonists: enhancement of leukotriene B4 synthesis. *Adv Exp Med Biol* 1991; 314: 73–89.
142 Antoine C, Murphy RC, Henson PM, Maclouf J. Time-dependent utilization of platelet arachidonic acid by the neutrophil in formation of 5-lipoxygenase products in platelet-neutrophil co-incubations. *Biochim Biophys Acta* 1992; 1128: 139–146.
143 Bigby TD, Meslier N. Transcellular lipoxygenase metabolism between monocytes and platelets. *J Immunol* 1989; 143: 1948–1954.
144 Maclouf JA, Murphy RC. Transcellular metabolism of neutrophil-derived leukotriene A4 by human platelets. A potential cellular source of leukotriene C4. *J Biol Chem* 1988; 263: 174–181.
145 Maugeri N, Evangelista V, Celardo A et al. Polymorphonuclear leukocyte-platelet interaction: role of P-selection in thromboxane B2 and leukotriene C4 cooperative synthesis. *Thromb Haemost* 1994; 72: 450–456.
146 Tornhamre S, Sjolinder M, Lindberg A et al. Demonstration of leukotriene-C4 synthase in platelets and species distribution of the enzyme activity. *Eur J Biochem* 1998; 251: 227–235.
147 Coeffier E, Delautier D, Le Couedic JP, Chignard M, Denizot Y, Benveniste J. Cooperation between platelets and neutrophils for paf-acether (platelet-activating factor) formation. *J Leukoc Biol* 1990; 47: 234–243.
148 Evangelista V, Celardo A, Dell'Elba G et al. Platelet contribution to leukotriene production in inflammation: in vivo evidence in the rabbit. *Thromb Haemost* 1999; 81: 442–448.
149 Serhan CN, Hamberg M, Samuelsson B. Lipoxins: novel series of biologically active compounds formed from arachidonic acid in human leukocytes. *Proc Natl Acad Sci USA* 1984; 81: 5335–5339.
150 Samuelsson B, Dahlen SE, Lindgren JA, Rouzer CA, Serhan CN. Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. *Science* 1987; 237: 1171–1176.
151 Edenius C, Haeggstrom J, Lindgren JA. Transcellular conversion of endogenous arachidonic acid to lipoxins in mixed human platelet–granulocyte suspensions [published

Cambridge Books Online © Cambridge University Press, 2009

erratum appears in *Biochem Biophys Res Commun* 1989 Feb 28; 159: 370]. *Biochem Biophys Res Commun* 1988; 157: 801–807.

152 Fiore S, Serhan CN. Formation of lipoxins and leukotrienes during receptor-mediated interactions of human platelets and recombinant human granulocyte/macrophage colony-stimulating factor-primed neutrophils. *J Exp Med* 1990; 172: 1451–1457.

153 Mayadas TN, Mendrick DL, Brady HR et al. Acute passive anti-glomerular basement membrane nephritis in P-selectin-deficient mice. *Kidney Int* 1996; 49: 1342–1349.

154 Larsen E, Celi A, Gilbert GE et al. PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. *Cell* 1989; 59: 305–312.

155 Fujimoto T, McEver RP. The cytoplasmic domain of P-selectin is phosphorylated on serine and threonine residues. *Blood* 1993; 82: 1758–1766.

156 Crovello CS, Furie BC, Furie B. Rapid phosphorylation and selective dephosphorylation of P-selectin accompanies platelet activation. *J Biol Chem* 1993; 268: 14590–14593.

157 Pytela R, Pierschbacher MD, Ginsberg MH, Plow EF, Ruoslahti E. Platelet membrane glycoprotein IIb/IIIa: member of a family of Arg-Gly-Asp—specific adhesion receptors. *Science* 1986; 231: 1559–1562.

158 Diacovo TG, deFougerolles AR, Bainton DF, Springer TA. A functional integrin ligand on the surface of platelets: intercellular adhesion molecule-2. *J Clin Invest* 1994; 94: 1243–1251.

159 Simon DI, Chen Z, Xu H et al. Platelet glycoprotein Ibalpha is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *J Exp Med* 2000; 192: 193–204.

160 Brown KK, Henson PM, Maclouf J, Moyle M, Ely JA, Worthen GS. Neutrophil–platelet adhesion: relative roles of platelet P-selectin and neutrophil beta2 (DC18) integrins. *Am J Respir Cell Mol Biol* 1998; 18: 100–110.

161 Schmidtke DW, Diamond SL. Direct observation of membrane tethers formed during neutrophil attachment to platelets or P-selectin under physiological flow. *J Cell Biol* 2000; 149: 719–730.

162 Palabrica T, Lobb R, Furie BC et al. Leukocyte accumulation promoting fibrin deposition is mediated in vivo by P-selectin on adherent platelets. *Nature* 1992; 359: 848–851.

163 Diacovo TG, Roth SJ, Buccola JM, Bainton DF, Springer TA. Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. *Blood* 1996; 88: 146–157.

164 Bengtsson T, Fryden A, Zalavary S, Whiss PA, Orselius K, Grenegard M. Platelets enhance neutrophil locomotion: evidence for a role of P-selectin. *Scand J Clin Lab Invest* 1999; 59: 439–449.

165 Coughlan AF, Hau H, Dunlop LC, Berndt MC, Hancock WW. P-selectin and platelet-activating factor mediate initial endotoxin-induced neutropenia. *J Exp Med* 1994; 179: 329–334.

166 Weyrich AS, McIntyre TM, McEver RP, Prescott SM, Zimmerman GA. Monocyte tethering by P-selectin regulates monocyte chemotactic protein-1 and tumor necrosis factor-alpha secretion. Signal integration and NF-kappa B translocation [see comments]. *J Clin Invest* 1995; 95: 2297–2303.

167 Yang J, Furie BC, Furie B. The biology of P-selectin glycoprotein ligand-1: its role as a selectin counterreceptor in leukocyte-endothelial and leukocyte–platelet interaction. *Thromb Haemost* 1999; 81: 1–7.

168 Larsen E, Palabrica T, Sajer S et al. PADGEM-dependent adhesion of platelets to monocytes and neutrophils is mediated by a lineage-specific carbohydrate, LNF III (CD15). *Cell* 1990; 63: 467–474.

169 Foxall C, Watson SR, Dowbenko D et al. The three members of the selectin receptor family recognize a common carbohydrate epitope, the sialyl Lewis^x oligosaccharide. *J Cell Biol* 1992; 117: 895–902.

170 Norgard KE, Moore KL, Diaz S et al. Characterization of a specific ligand for P-selection on myeloid cells. A minor glycoprotein with sialylated O-linked oligosaccharides [published erratum appears in *J Biol Chem* 1993 Oct 25; 268: 22953]. *J Biol Chem* 1993; 268: 12764–12774.

171 Pouyani T, Seed B. PSGL-1 recognition of P-selectin is controlled by a tyrosine sulfation consensus at the PSGL-1 amino terminus. *Cell* 1995; 83: 333–343.

172 Tang T, Frenette PS, Hynes RO, Wagner DD, Mayadas TN. Cytokine-induced meningitis is dramatically attenuated in mice deficient in endothelial selectins. *J Clin Invest* 1996; 97: 2485–2490.

173 Mulligan MS, Watson SR, Fennie C, Ward PA. Protective effects of selectin chimeras in neutrophil-mediated lung injury. *J Immunol* 1993; 151: 6410–6417.

174 Borges E, Eytner R, Moll T et al. The P-selectin glycoprotein ligand-1 is important for recruitment of neutrophils into inflamed mouse peritoneum. *Blood* 1997; 90: 1934–1942.

175 Takada M, Nadeau KC, Shaw GD, Marquette KA, Tilney NL. The cytokine-adhesion molecule cascade in ischemia/reperfusion injury of the rat kidney. Inhibition by a soluble P-selectin ligand. *J Clin Invest* 1997; 99: 2682–2690.

176 Stone JP, Wagner DD. P-selectin mediates adhesion of platelets to neuroblastoma and small cell lung cancer. *J Clin Invest* 1993; 92: 804–813.

177 Dardik R, Savion N, Kaufmann Y, Varon D. Thrombin promotes platelet-mediated melanoma cell adhesion to endothelial cells under flow conditions: role of platelet glycoproteins P-selectin and GPIIb-IIIA. *Br J Cancer* 1998; 77: 2069–2075.

178 Weber C, Springer TA. Neutrophil accumulation on activated, surface-adherent platelets in flow is mediated by interaction of Mac-1 with fibrinogen bound to alphaIIbbeta3 and stimulated by platelet-activating factor. *J Clin Invest* 1997; 100: 2085–2093.

179 Ostrovsky L, King AJ, Bond S et al. A juxtacrine mechanism for neutrophil adhesion on platelets involves platelet-activating factor and a selectin-dependent activation process. *Blood* 1998; 91: 3028–3036.

180 Zhou W, Javors MA, Olson MS. Platelet-activating factor as an

Cambridge Books Online © Cambridge University Press, 2009

intercellular signal in neutrophil-dependent platelet activation. *J Immunol* 1992; 149: 1763–1769.
181 Walzog B, Weinmann P, Jeblonski F, Scharffetter-Kochanek K, Bommert K, Gaehtgens P. A role for beta(2) integrins (CD11/CD18) in the regulation of cytokine gene expression of polymorphonuclear neutrophils during the inflammatory response. *FASEB J* 1999; 13: 1855–1865.
182 Evangelista V, Manarini S, Rotondo S et al. Platelet/polymorphonuclear leukocyte interaction in dynamic conditions: evidence of adhesion cascade and cross talk between P-selectin and the beta 2 integrin CD11b/CD18. *Blood* 1996; 88: 4183–4194.
183 Coller BS. Platelet GPIIb/IIIa antagonists: the first anti-integrin receptor therapeutics. *J Clin Invest* 1997; 99: 1467–1471.
184 Xiao Z, Theroux P, Frojmovic M. Modulation of platelet-neutrophil interaction with pharmacological inhibition of fibrinogen binding to platelet GPIIb/IIIa receptor. *Thromb Haemost* 1999; 81: 281–285.
185 Bombeli T, Schwartz BR, Harlan JM. Adhesion of activated platelets to endothelial cells: evidence for a GPIIbIIIa-dependent bridging mechanism and novel roles for endothelial intercellular adhesion molecule 1 (ICAM-1), alphavbeta3 integrin, and GPIbalpha. *J Exp Med* 1998; 187: 329–339.
186 Coller BS. Binding of abciximab to alpha V beta 3 and activated alpha M beta 2 receptors: with a review of platelet–leukocyte interactions. *Thromb Haemost* 1999; 82: 326–336.
187 Ginsberg MH, Kozin F, O'Malley M, McCarty DJ. Release of platelet constituents by monosodium urate crystals. *J Clin Invest* 1977; 60: 999–1007.
188 Barabe F, Gilbert C, Liao N, Bourgoin SG, Naccache PH. Crystal-induced neutrophil activation VI. Involvment of FcgammaRIIIB (CD16) and CD11b in response to inflammatory microcrystals. *FASEB J* 1998; 12: 209–220.
189 Sitrin RG, Pan PM, Srikanth S, Todd RF, 3rd. Fibrinogen activates NF-kappa B transcription factors in mononuclear phagocytes. *J Immunol* 1998; 161: 1462–1470.
190 Mocsai A, Ligeti E, Lowell CA, Berton G. Adhesion-dependent degranulation of neutrophils requires the Src family kinases Fgr and Hck. *J Immunol* 1999; 162: 1120–1126.
191 Evangelista V, Manarini S, Sideri R et al. Platelet/polymorphonuclear leukocyte interaction: P-selectin triggers protein–tyrosine phosphorylation-dependent CD11b/CD18 adhesion: role of PSGL-1 as a signaling molecule. *Blood* 1999; 93: 876–885.
192 Zatta A, Prosdocimi M, Bertele V, Bazzoni G, Del Maschio A. Inhibition of platelet function by polymorphonuclear leukocytes. *J Lab Clin Med* 1990; 116: 651–660.
193 Levine PH, Weinger RS, Simon J, Scoon KL, Krinsky NI. Leukocyte platelet interaction. Release of hydrogen peroxide by granulocytes as a modulator of platelet reactions. *J Clin Invest* 1976; 57: 955–963.
194 Salvemini D, de Nucci G, Gryglewski RJ, Vane JR. Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor. *Proc Natl Acad Sci USA* 1989; 86: 6328–6332.
195 Brower MS, Levin RI, Garry K. Human neutrophil elastase modulates platelet function by limited proteolysis of membrane glycoproteins. *J Clin Invest* 1985; 75: 657–666.
196 Valles J, Santos MT, Marcus AJ et al. Downregulation of human platelet reactivity by neutrophils. Participation of lipoxygenase derivatives and adhesive proteins. *J Clin Invest* 1993; 92: 1357–1365.
197 Marcus AJ, Safier LB. Thromboregulation: multicellular modulation of platelet reactivity in hemostasis and thrombosis. *FASEB J* 1993; 7: 516–522.
198 Rinder HM, Tracey JL, Rinder CS, Leitenberg D, Smith BR. Neutrophil but not monocyte activation inhibits P-selectin-mediated platelet adhesion. *Thromb Haemost* 1994; 72: 750–756.
199 Nicolini FA, Wilson AC, Mehta P, Mehta JL. Comparative platelet inhibitory effects of human neutrophils and lymphocytes. *J Lab Clin Med* 1990; 116: 147–152.
200 Ginsburg I, Quie PG. Modulation of human polymorphonuclear leukocyte chemotaxis by leukocyte extracts, bacterial products, inflammatory exudates, and polyelectrolytes. *Inflammation* 1980; 4: 301–311.
201 Jancinova V, Drabikova K, Nosal R, Danihelova E. Platelet-dependent modulation of polymorphonuclear leukocyte chemiluminescence. *Platelets* 2000; 11: 278–285.
202 Wilson E, Laster SM, Gooding LR, Lambeth JD. Platelet-derived growth factor stimulates phagocytosis and blocks agonist-induced activation of the neutrophil oxidative burst: a possible cellular mechanism to protect against oxygen radical damage. *Proc Natl Acad Sci USA* 1987; 84: 2213–2217.
203 McGarrity ST, Stephenson AH, Hyers TM, Webster RO. Inhibition of neutrophil superoxide anion generation by platelet products: role of adenine nucleotides. *J Leukoc Biol* 1988; 44: 411–421.
204 Wong CS, Gamble JR, Skinner MP, Lucas CM, Berndt MC, Vadas MA. Adhesion protein GMP140 inhibits superoxide anion release by human neutrophils. *Proc Natl Acad Sci USA* 1991; 88: 2397–2401.
205 McGarrity ST, Hyers TM, Webster RO. Inhibition of neutrophil functions by platelets and platelet-derived products: description of multiple inhibitory properties. *J Leukoc Biol* 1988; 44: 93–100.
206 Maderna P, Colli S, Caruso D et al. Quantitative changes of hydroxyacid formation during platelet-neutrophil interaction. *J Lab Clin Med* 1993; 121: 406–414.
207 Andonegui G, Trevani AS, Lopez DH, Raiden S, Giordano M, Geffner JR. Inhibition of human neutrophil apoptosis by platelets. *J Immunol* 1997; 158: 3372–3377.
208 Brunetti M, Martelli N, Manarini S et al. Polymorphonuclear leukocyte apoptosis is inhibited by platelet-released mediators, role of TGFbeta-1 [In Process Citation]. *Thromb Haemost* 2000; 84: 478–483.
209 Del Maschio A, Bazzoni G, Dejana E. Polymorphonuclear leukocyte adhesion to endothelial cells is inhibited by resting platelets. *Arterioscler Thromb* 1992; 12: 1420–1427.
210 Maione TE, Gray GS, Petro J et al. Inhibition of angiogenesis

Cambridge Books Online © Cambridge University Press, 2009

by recombinant human platelet factor-4 and related peptides. *Science* 1990; 247: 77–79.
211 Broxmeyer HE, Sherry B, Cooper S et al. Comparative analysis of the human macrophage inflammatory protein family of cytokines (chemokines) on proliferation of human myeloid progenitor cells. Interacting effects involving suppression, synergistic suppression, and blocking of suppression. *J Immunol* 1993; 150: 3448–3458.
212 Gentilini G, Kirschbaum NE, Augustine JA, Aster RH, Visentin GP. Inhibition of human umbilical vein endothelial cell proliferation by the CXC chemokine, platelet factor 4 (PF4), is associated with impaired downregulation of p21(Cip1/WAF1). *Blood* 1999; 93: 25–33.
213 O'Meara YM, Brady HR. Lipoxins, leukocyte recruitment and the resolution phase of acute glomerulonephritis. *Kidney Int Suppl* 1997; 58: S56–S61.

Cambridge Books Online © Cambridge University Press, 2009

58

Platelets and the preimplantation stage of embryo development

Christopher O'Neill

Human Reproduction Unit, Department of Physiology, University of Sydney, Royal North Shore Hospital, St Leonards NSW, Australia

Introduction

The preimplantation phase of pregnancy occurs from the time of fertilization until the embryo implants into the endometrium of the uterus. Implantation occurs on days 5–6 of pregnancy in humans. Implantation triggers differentiation of the embryo's trophoblast layer, leading to a sequence of developmental changes that produce the placenta. Prior to implantation, the embryo exists in a free-living state, initially within the oviduct (fallopian tube) and then in the lumen of the uterus. It has generally been considered that the embryo is passive during this time, relying upon the reproductive tract for the provision of its nutrients and a benevolent environment. However, it is now becoming clear that the local environment of the reproductive tract is conditioned and modified by the early embryo and these modifications are important for normal embryo development. These modifications include the activation of blood platelets within the reproductive tract and are elicited by the production of soluble and diffusible factors by the early embryo. The best characterized of these is platelet-activating factor (PAF).

Embryo-derived platelet-activating factor

The well-known ether phospholipid 1-*o*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphocholine (PAF) is a potent pleiotrophic mediator (Fig. 58.1). It is produced by the preimplantation embryo of many species soon after fertilization (mouse[1,2]; human[3,4]; rabbit[5]; and sheep[6]). It accumulates to significant concentrations within the embryo and is also released into the embryo's surrounding fluid. The production of embryo-derived PAF seems to be largely *de novo* occurring from simple substrates[7,8]. The rate-limiting enzyme for the synthesis of embryo-derived PAF is

$n = 15, 17$

Fig. 58.1. The structure of platelet-activating factor.

lysoPAF: acetyltransferase[8] (EC 2.3.1.67). This calcium-dependent enzyme is present within the preimplantation embryo. Its activity is at a low level in the unfertilized oocyte but increases soon after fertilization. This is the time when PAF release from the zygote is first detected[9]. This temporal correlation between the activity of lysoPAF: acetyltransferase and PAF release suggests that activation of this rate-limiting enzyme is the regulatory event following fertilization that initiates PAF production and release by the early embryo. The activation of lysoPAF: acetyltransferase activity and PAF release occur prior to the onset of new transcription of mRNA from the zygotic genome[10]. Thus, the production of PAF is not dependent upon transcription but on the post-transcriptional activation of the biosynthetic pathway.

The release of PAF by the preimplantation embryo was first detected by the observation that a mild but consistent thrombocytopenia occurred in mice within a short time of fertilization and persisted throughout the preimplantation phase of pregnancy[11]. The thrombocytopenia was unequivocally a consequence of the presence of the preimplantation embryo[11,12]. It has been subsequently shown that spermatozoa[13], the luteal phase uterus[14,15] and follicular fluid[16] also produce PAF, yet these sources of PAF are without detectable effect on blood platelets and do not induce thrombocytopenia.

Early pregnancy-induced thrombocytopenia

The release of PAF from the embryo induces localized platelet activation within the vasculature of the reproductive tract[17]. It is assumed that these activated platelets are trapped by the reticuloendothelial system and removed from the circulation, resulting in thrombocytopenia[11]. In the mouse, this platelet activation is accompanied by the contraction of the spleen[11] and preferential loss of platelets from the spleen into circulation. This is a well-characterized response to increased platelet activation – with the spleen acting as a reservoir of blood platelets that can be called upon during acute periods of platelet consumption[18,19]. This homeostatic mechanism normally acts to stabilize the number of platelets in the circulation; only prolonged or profound loss of platelets results in detectable peripheral thrombocytopenia. The size of the spleen's platelet reservoir varies greatly between species[18]. The consistent detection of thrombocytopenia in mice is testament to the extent that PAF released by the embryo causes platelet activation. The mouse, however, may be a special case – thrombocytopenia may not be a general characteristic of early pregnancy in all mammalian species.

Thrombocytopenia was observed in the preimplantation phase of some human pregnancies[20], but in others there was a trend to thrombocytosis[21]. The variable response may reflect the ability of the spleen to compensate for platelet loss from the circulation in the human, or it may be a consequence of increased thrombopoiesis compensating for platelet loss in early human pregnancy. An association was discovered between the thrombocrit prior to pregnancy and the change in platelet count during early pregnancy. Those women with a high thrombocrit prior to pregnancy showed a subsequent thrombocytopenia. By contrast, those with a low thrombocrit generally did not display thrombocytopenia. A possible interpretation of this result is that release of the splenic platelet pool or activation of thrombopoiesis occurred more readily and rapidly where the thrombocrit, prior to pregnancy, was low[21]. Early pregnancy was also associated with variable thrombocytopenia in cows following embryo transfer[22]. It is noteworthy that, in the intact female rabbit, thrombocytopenia in early pregnancy is not readily detected[23]. However, in splenectomized females, early pregnancy associated thrombocytopenia was detected[23]. This observation supports the role of the spleen in the homeostatic maintenance of the circulating platelet concentration following the localized platelet activation that occurs during early pregnancy. A further consideration is that the mouse is a polytocous species, therefore many embryos are present in a small reproductive tract. This contrasts with the human, where generally only one embryo is present in a large reproductive tract (with a correspondingly large blood supply). Thus in large mono-ovulatory species, even extensive early pregnancy-associated platelet activation may be less likely to be detected as peripheral thrombocytopenia. It seems that monitoring platelet numbers during early pregnancy cannot be used in a reliable manner to detect or monitor pregnancy in most species[21]. Should reliable, sensitive and accurate measures of platelet turnover become widely available, these may prove useful for monitoring early pregnancy.

Metabolism of PAF and platelet activation in the reproductive tract

PAF is present in many reproductive tissues (sperm[13], ovary[16], uterus[14]), yet PAF-induced platelet activation and transient thrombocytopenia is only associated with early pregnancy[11,12]. The current assay methods do not allow reliable measurement of the rate of PAF release but only the amount that accumulates in medium over time. The zygote releases 1.3–6.7 ng PAF/embryo (mouse) and 0–80 ng PAF/embryo (human)[24,25] over a 12 h period. It is not known if this is released at a consistent rate over this period, or whether its release is episodic.

There exists in most tissues and fluids studied to date a high capacity enzyme PAF: acetylhydrolase, that degrades PAF to its inactive metabolite, lysoPAF. It was anticipated that, within the reproductive tract, there would be sufficient PAF: acetylhydrolase activity to degrade all the embryo-derived PAF released prior to it being able to cause significant platelet activation. The fact that extensive platelet activation was observed to occur suggested that there was some interesting PAF biology occurring within the reproductive tract. Acute administration of synthetic PAF at a concentration at least an order of magnitude higher than is produced by the embryo was required to induce detectable thrombocytopenia in the mouse, and this thrombocytopenia was only transient despite the high doses[12].

Since embryo-derived PAF was unequivocally demonstrated to be structurally homologous with synthetic-PAF[2], studies were performed to determine whether the uterine environment resulted in reduced metabolism of PAF[26]. The activity of PAF: acetylhydrolase was measured in uterine tissue and in uterine lumen fluid throughout the reproductive cycle and in early pregnancy. A precipitous decline in enzyme activity in these preparations occurred after ovulation. This resulted in both uterine tissue and lumenal

Cambridge Books Online © Cambridge University Press, 2009

fluid having low enzyme activity during the preimplantation phase[26]. PAF: acetylhydrolase activity in the uterus increased substantially after implantation. These changes in the enzyme activity occurred without detectable alterations to plasma enzyme activity. Thus, during the period that the preimplantation embryo released PAF the catalytic activity directed against PAF in the uterus declined to a low level. The change in enzyme activity would be expected to result in a prolonged half-life of embryo-derived PAF. The decline in PAF: acetylhydrolase activity was steroid hormone dependent, with progesterone dominance over estradiol being required[26]. This is the pattern of hormone production during the preimplantation (early luteal) phase of pregnancy.

Investigations show that the plasma form of PAF: acetylhydrolase is responsible for the activity in the uterus (O. Chami & C. O'Neill, unpublished data). It seems that hormone-dependent alterations in blood flow and vasopermability in the uterus allow dynamic and rapid alterations in the PAF: acetylhydrolase activity within the uterus independently of a change in enzyme activity within the blood plasma. The results suggest that a reduction in PAF metabolism within the uterus during the preimplantation phase may contribute to the ability of the small amounts of PAF released by the embryo to induce platelet activation.

Another mechanism favouring a prolonged half-life for embryo-derived PAF also exists in the uterus. Recent results show that PAF released by the embryo is released in a unique binding configuration with albumin that prevents or dramatically reduces its metabolism by PAF: acetylhydrolase[27,28]. A preliminary observation in support of this hypothesis was that PAF released from human embryos in medium containing high concentrations of human serum ($<$10% v/v) was stable[29], yet the culture medium contained a high concentration of PAF: acetylhydrolase from the serum[30]. When an equivalent concentration of synthetic PAF was added to the same embryo culture medium, it was degraded within minutes by the PAF: acetylhydrolase present in the serum[30].

The possibility that, upon its release from cells, PAF was bound to a special protein that protected it from metabolism was tested. However, PAF released from embryos was all bound to albumin[27,28]. Albumin in medium was required for PAF release by embryos and PAF co-migrated with albumin upon various forms of chromatography. An alternative hypothesis was tested: the nature of albumin's binding of embryo-derived PAF conferred protection from metabolism by PAF: acetylhydrolase. When embryo-conditioned medium was subjected to organic extraction and the resulting purified PAF was then added back to medium containing albumin, the PAF became readily susceptible to the action of PAF: acetylhydrolase[27,28]. In this regard its behaviour was equivalent to synthetic-PAF added to medium. Extracted embryo-derived PAF and synthetic-PAF added to medium bound preferentially to hydrophobic surfaces, being lost from the albumin. By contrast, unextracted embryo-derived PAF remained bound to albumin in the presence of hydrophobic surfaces. Both synthetic PAF and extracted embryo-derived PAF, when added to embryo culture medium, could be recognized by a PAF-specific antibody and could thus be measured by a direct immunoassay. This was not the case for PAF in embryo-conditioned medium from which PAF could only be assayed after its organic extraction. The results suggest that upon the release of PAF from embryos (but not following its addition to medium) PAF binds to albumin in a manner that protects it from metabolism by PAF: acetylhydrolase. The fact that this type of binding to albumin also prevented a PAF antibody binding to embryo-derived PAF suggests that it may involve a steric modification of the albumin molecule[27,28].

Limited proteolytic analysis of albumin showed that PAF released from cells bound only to a site within domain II of the albumin molecule (total of 583 amino acids), between amino acids 240 and 386[27,28]. By contrast, when PAF was added to medium it bound to albumin at sites other than domain II. Albumin forms a heart-shaped molecule of three domains. Domain II (amino acid 208–398) is composed of two similar subdomains, *a* and *b*, each being composed of a cluster of four α-helices[31]. The interface between subdomains II*a* and II*b* form a hydrophobic cleft that may serves as a binding site for lipids[31].

The observation that PAF binding within domain II only occurred upon release of PAF from cells but not upon addition of PAF to albumin-containing medium, infers that an interaction between the cell and albumin is required before PAF can be loaded onto domain II of albumin. Covalent modification of albumin involving cysteine–cysteine disulfide bonds seemed to be required for PAF to be loaded onto domain II from cells. For instance, after exposure to cells there was an increased molar concentration of solvent exposed thiol groups; albumin was more susceptible to denaturation following exposure to dithiothreitol; and following such denaturation, the albumin-associated PAF became susceptible to degradation by PAF: acetylhydrolase[27,28]. Notwithstanding the high affinity nature of this binding, the PAF released was clearly still active as assessed by the platelet activation and thrombocytopenia induced in early pregnancy and the thrombocytopenia induced in mice upon injection of embryo-conditioned culture medium[12].

While this special form of cell-dependent release of PAF

Cambridge Books Online © Cambridge University Press, 2009

within domain II of albumin was first observed with preimplantation stage embryos, it was not restricted to this type of cell. The same biochemical behaviour was observed for PAF released by endothelial cells[28]. Thus PAF may be released from cells in at least two forms. It can be released and bound in a superficial manner with albumin. In this configuration it can be rapidly degraded by PAF: acetylhydrolase. It consequently has a short half-life and can act only as a local mediator. PAF released from inflammatory cells probably exists primarily in this configuration. For other cells in non-inflammatory states that release PAF, such as the embryo and endothelial cells, PAF can be released in a stable configuration in association with albumin. It can accumulate due to its prolonged half-life and can therefore enter the circulation. It may therefore have roles as a conventional circulating hormone, as well as a local mediator. These important implications arising from studies of embryo-derived PAF warrant further investigation.

Physiological actions of embryo-derived PAF

A range of PAF antagonists can inhibit the thrombocytopenia induced by embryo-derived PAF[32]. When administered throughout the preimplantation phase of pregnancy, PAF antagonists also blocked the successful implantation of embryos in rats and mice[32–35]. The pharmacology of these antagonists was curious, however. The dose–response showed a characteristic quadratic effect, with inhibition of pregnancy at relatively low doses but loss of the inhibition at higher doses of antagonist[36].

This characteristic quadratic dose–response occurred for a range of antagonists with varying structures, and was independent of its mode or route of administration[36]. The nature of the dose–response suggests that the antagonists act as partial agonists rather than as true antagonists. This mode of action has not been widely reported or observed in other PAF mediated processes, although WEB 2086 and WEB 2170[37] are reported to exert partial agonistic actions on the perfused heart. PAF antagonists can also act as inhibitors of PAF metabolism[38], thus the complex pharmacological response observed at high drug concentrations in early pregnancy may result from the dual interactions of the drugs with the PAF-receptor and PAF: acetylhydrolase. It is therefore of interest to note that the genetic knockout of the β-subunit of intracellular PAF: acetylhydrolase 1b also results in implantation failure and early embryonic demise[39]. The observation that PAF antagonists act as true antagonists in many models but partial agonists in others suggests that they may be acting against different receptor types. Another possibility may be that the binding of PAF within domain II of albumin alters the nature of its interaction with the PAF-receptor and hence alters the pharmacology of the PAF antagonists. Currently, there is insufficient experimental data to distinguish between these alternative hypotheses.

Using the antagonists in a dose range where pregnancy was inhibited, embryo transfer experiments established that the actions of embryo-derived PAF are required for the normal development of the embryo[40]. There was no support for a role of embryo-derived PAF in the preparation of the maternal reproductive tract to be receptive to the embryo. These embryo transfer experiments with PAF antagonist treated mice did not, however, establish whether this action of PAF was a direct autocrine effect or whether it was indirect – for instance, secondary to the activation of platelets. There is experimental data to support a role for both mechanisms.

Roles for platelet activation during early pregnancy

A factor of maternal origin that is expressed with a similar temporal profile to PAF is a protein known as early pregnancy factor (EPF)[41]. For many years this factor was known by its actions in modifying T-lymphocyte function. Its secretion during early pregnancy was reasoned to provide an immunosuppressive action that allowed the embryonic 'allograft' to survive the maternal immune system. It was recently shown that PAF induces the expression of EPF[42], while EPF has been shown to be a secreted form of chaperonin 10[43]. It is released from blood platelets following their activation[43]. Chaperonin 10 is normally considered an intracellular protein that has important functions in protein folding and conformation. It acts in concert with chaperonin 60[44] to allow importation of proteins into cellular organelles, particularly mitochondria, and is therefore essential for normal organellogenesis.

The discovery of chaperonin 10 as a PAF-induced secretory product of platelets, that is present in the blood of pregnant animals, was an unexpected observation. A full description of chaperonin 10's actions in early pregnancy is awaited. However, it may exert actions in modifying the conformation of some extracellular proteins or perhaps has a role in protein import into cells. The observation that immunization of mice against EPF resulted in failed embryo development and pregnancy loss[45] demonstrates the important role of the factor in normal pregnancy.

A confounding observation was that, while synthetic-PAF induced chaperonin 10 release from platelets in vitro,

Cambridge Books Online © Cambridge University Press, 2009

embryo-derived PAF apparently did not[46]. This conflicting result raised questions about the role of embryo-derived PAF in chaperonin 10 release from platelets. The experimental design and conclusion, however, did not take into account the evidence that embryo-derived PAF is not active in defined medium in vitro because of it binding within domain II of albumin. It is also not capable of inducing blood platelet activation in vitro[27,28] yet upon injection into mice it does induce platelet activation and thrombocytopenia[1]. This difference between its actions in vitro and in vivo suggests that an auxiliary activity may also be required to allow embryo-derived PAF accessible to its receptor when it is bound to domain II of albumin. Since binding in this configuration involves modifications to cysteine–cysteine disulfide bonds[27,28], it seems likely that a protein disulfide isomerase or thioredoxin-like activity may be required.

The expression of EPF activity requires the action of a circulating thioredoxin[47]. Indeed, it was shown that pregnancy is associated with the expression of a novel secreted form of thioredoxin[48]. The target of action of this thioredoxin is yet to be defined. We speculate that the secreted thioredoxin may change the conformation of albumin that contains embryo-derived PAF within domain II. Following this conformational change to albumin, PAF can access its receptor allowing it to activate maternal blood platelets and other target cells. The platelets release their contents, including chaperonin 10.

A full description of the actions and interactions of these four players – PAF, albumin, secreted thioredoxin and secreted chaperonin 10 – will provide a new model for intercellular communication. The observation that a similar form of secreted chaperonin 10 is also active in neoplastic tissue and in rapidly proliferating and regenerating tissues suggest a fundamental role for this novel peptide[44]. The platelet, as a rich source of secreted chaperonin 10, may have a hitherto unexpected role in intracellular communication and regulation.

The actions of PAF on the oviduct

As well as acting on platelets, embryo-derived PAF may act directly on the oviduct. The gene coding for a G-protein linked receptor for PAF is expressed on the epithelial and subepithelial cells of the hamster oviduct. Oviductal cells (cow) in primary culture responded to PAF with a characteristic calcium transient that required extracellular calcium and was receptor dependent[49]. The responsiveness of the cells varied with the stage of the estrous cycle. Treatment with PAF could also induce proliferation in these cells[49]. In the human, PAF induced an increase in the transepithelial potential difference across a polarized primary culture layer of oviductal epithelial cells and also increased the shortcircuit current activity. The results were interpreted to indicate an effect of PAF on the transepithelial Cl^- current and hence regulate oviductal fluid production[50].

It has been also shown that embryo-derived PAF provides a message that allows the reproductive tract to distinguish between fertilized and unfertilized oocytes, providing a means of differential transport[51] (fertilized oocytes are transported down the oviduct towards the uterus, while unfertilized oocytes remain trapped within the upper oviduct where they degenerate). It has been suggested that PAF's ability to regulate fluid fluxes across the epithelium may be a mechanism for its role in embryo migration[50]. Embryo-derived PAF also induces a reduction of blood flow to the oviduct due to a decrease in the median cross-sectional area of blood vessels (indicating vasoconstriction) and the collapse of subepithelial capillaries[17]. Embryo-derived PAF also induces a reduction in the incidence of fenestrated capillaries in the oviduct (that are common in pseudopregnant animals)[17]. This alteration in the pattern of fenestrations may act together with PAF's transepithelial fluid flow to regulate oviduct fluid dynamics. It has not yet been defined whether these actions of embryo-derived PAF on vascular dynamics are direct or indirect effects, for instance as a consequence of localized platelet activation. Fig. 58.2 shows the possible interrelationships of these actions.

Action of PAF released by the uterus in early pregnancy

During early pregnancy, PAF is produced by the uterus as well as the embryo. The steroid hormones of the luteal phase cause a significant increase in the synthesis of PAF by the uterus[14,15,52,53]. Although endometrial PAF is released, and can be detected within the uterine lumen[53], it does not appear to result in detectable platelet activation[11]. The PAF that is released is not bound to domain II of albumin and is readily degraded by PAF: acetylhydrolase[53]. Therefore, unlike embryo-derived PAF, it is unlikely to accumulate to sufficient concentrations to enter the blood vessels to induce platelet activation. Within the uterus, the main role of PAF seems to be to modify the production and release of prostaglandins[54,55]. In sheep, a good case has been made for PAF produced by the uterus to act as an autocrine pulse-generator of luteolytic $PGF_{2\alpha}$[53]. The pulsatile release of $PGF_{2\alpha}$ acts on the corpus luteum of the ovary

Cambridge Books Online © Cambridge University Press, 2009

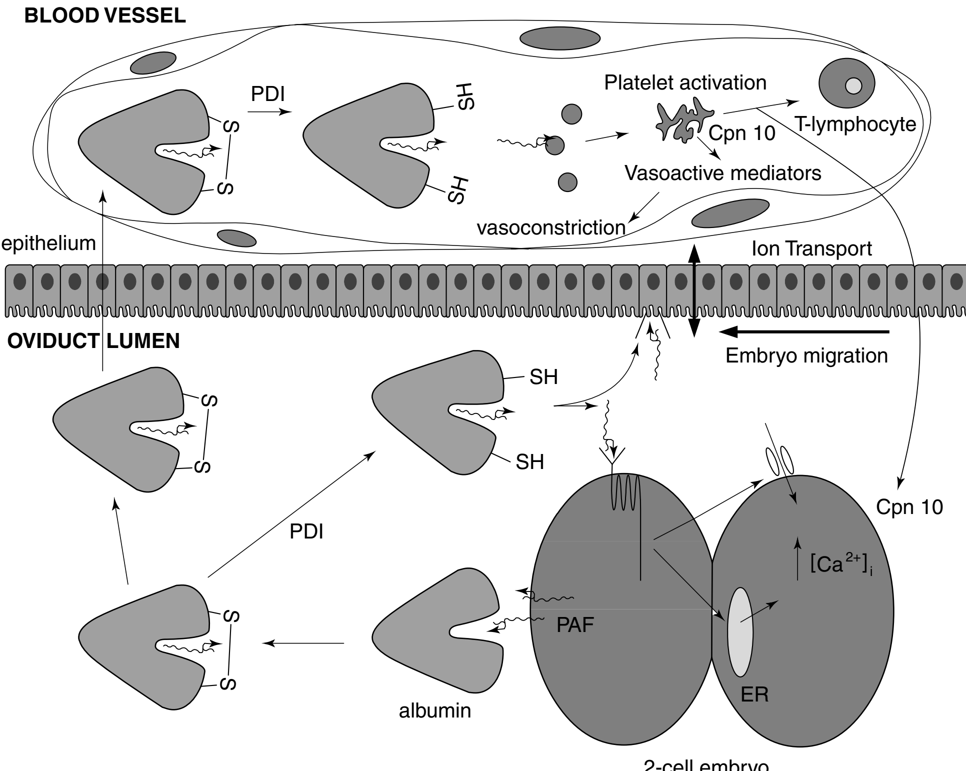


Fig. 58.2. A schematic representation of hypothetical interactions of embryo-derived PAF. ER = endoplasmic reticulum; $[Ca^{2+}]_i$ = intracellular calcium ion concentration; PDI = protein disulfide isomerase; Cpn10 = chaperonin 10.

to prevent the secretion of progesterone. During pregnancy, embryonic factors (interferon tau in the sheep) act to block this pulse-generation by the embryo (Fig. 58.3). It is yet to be determined whether these actions occur via PAF's action on platelets or by direct action on the cells of the endometrium.

Autocrine actions of embryo-derived PAF

As well as the actions of embryo-derived PAF on the reproductive tract, it also has an important role as an autocrine mediator of embryo development. PAF can act directly upon the embryo to enhance its rate of oxidative metabolism[56] and survival[57,58]. Embryos cultured in defined medium containing PAF grow to the blastocyst stage with greater cell numbers[59–61]. The transfer of embryos treated in this way resulted in increased implantation and birth rates[57,62]. The production of zygotes by the method of in vitro fertilization reduces the ability of embryos to release PAF[60]. This is correlated with reduced growth and viability of the IVF embryos. Supplementation of medium with PAF improves the survival and viability of the resulting embryos without having specific growth factor effects[61], while an antibody to PAF blocked embryo development[63].

PAF acts on the zygote and two-cell stage embryo by specifically inducing intracellular calcium transients[64,65]. These transients can be induced by both embryo-derived and exogenous PAF[65]. The calcium transients occur as single global waves with a periodicity of approximately 60–90 minutes[65]. The transients result from an interdependent combination of the release of intracellular stores of calcium and the influx of external calcium. Blocking the calcium transients blocked normal embryo development, showing that this is the important mechanism of signal transduction for PAF in the preimplantation embryo. Extracellular albumin is required for PAF release and the actions of PAF can be prevented by pretreatment of embryos with high concentrations of PAF: acetylhydrolase. The results to date show embryo-derived PAF acts as an autocrine trophic mediator but do not provide support for its actions as an intracellular message or as a juxtacrine mediator in the early embryo[65].

Cambridge Books Online © Cambridge University Press, 2009

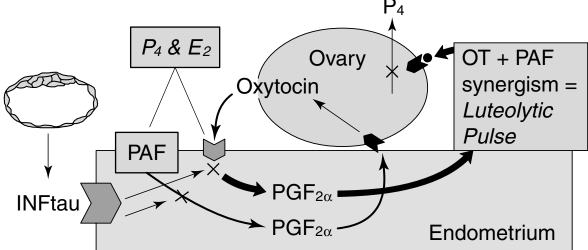


Fig. 58.3. A schematic representation of hypothetical interactions of endometrial PAF leading to luteolytic pulses of $PGF_{2\alpha}$ in the sheep. P_4 = progesterone; E_2 = estradiol 17α; OT = oxytocin.

Conclusions

Platelet-activating factor coordinates a range of important physiological processes during early pregnancy. Its release occurs in a novel manner, being bound to serum albumin in a 'caged' form. This form of binding is associated with conformational alteration of the albumin molecule involving cysteine–cysteine disulfide bonds. In this configuration PAF is resistant to metabolism by PAF: acetylhydrolase, shifting its mode of action from a labile local mediator to a stable mediator acting on various tissues. An important target is the embryo itself, with released PAF acting in an autocrine loop. It exerts paracrine effects, inducing extensive blood platelet activation and altered vascular dynamics within the reproductive tract. Embryo-derived PAF also signals to the oviduct to induce embryo transport down the fallopian tube to the uterus, perhaps via modification of oviductal fluid formation. This provides a means of differential migration for fertilized and unfertilized eggs. Platelet activation results in the release of a novel secreted form of chaperonin 10. This protein probably has a role in modifying protein conformation within the embryo's vicinity, including those associated with T-lymphocyte function. Although its precise function in early pregnancy is yet to be defined, its role in embryo development is established by the pregnancy failure that occurs following immunisation against it. The release and action of chaperonin 10 from platelets adds a further interesting candidate in the long list of potent cellular mediators released by platelets.

References

1 O'Neill C. Partial characterisation of the embryo-derived platelet activating factor in mice. *J Reprod Fertil* 1985; 75: 375–380.

2 Kodama H, Muto H, Maki M. Isolation and identification of embryo-derived platelet-activating factor in mice. *Acta Obst Gyn Jpn* 1989; 41: 899–906.

3 Collier M, O'Neill C, Ammit AJ, Saunders DM. Biochemical and pharmacological characterisation of human embryo-derived platelet activating factor. *Human Reprod* 1988; 3: 993–998.

4 Vereecken A, Delbeke L, Angle M, Gielis M, Gerris J, Johnston J, Buytaert P. Embryo-derived platelet activating factor, a marker of embryo quality and viability following ovarian stimulation for in vitro fertilisation. *J In Vitro Fertil Emb Transfer* 1990; 7: 321–326.

5 Minhas BS, Zhu Y, Kim H, Burwinkel TH, Ripps BA, Buster JE. Embryonic platelet activating factor production in the rabbit increases during the preimplantation phase. *J Assist Reprod Genet* 1993; 10: 366–370.

6 Battye KM, Ammit AJ, O'Neill C, Evans G. Production of platelet-activating factor by the preimplantation sheep embryo. *J Reprod Fertil* 1991; 93: 507–514.

7 Wells XE, O'Neill C. Biosynthesis of platelet-activating factor by the mouse two-cell embryo. *J Reprod Fertil* 1992; 96: 61–71.

8 Wells XE, O'Neill C. Detection and preliminary characterization of two enzymes involved in the biosynthesis of platelet-activating factor in mouse oocytes, zygotes and preimplantation embryos: dithiothreitol-insensitive cytidinediphospho-choline: 1-*o*-alkyl-2-acetyl-*sn*-glycerol cholinephosphotransferase and acetyl-coenzyme A:1-*o*-alkyl-2-lyso-sn-glycero-3-phosphocholine acetyltransferase. *J Reprod Fertil* 1994; 101: 385–391.

9. Gross TS, Thatcher WW, O'Neill C. Platelet-activating factor alters the dynamics of prostaglandin and protein synthesis by endometrial explants from pregnant and cyclic cows at day 17 following estrus. *Theriogenology* 1990; 34: 205–218.

10 Schultz RM. Regulation of zygotic gene activation in the mouse. *BioEssays* 1993; 15: 531–538.

11 O'Neill C. Thrombocytopenia is an initial maternal response to fertilisation in mice. *J Reprod Fertil* 1985; 73: 559–566.

12 O'Neill C. Examination of the causes of early pregnancy associated thrombocytopenia in mice. *J Reprod Fertil* 1985; 73: 567–577.

13 Kumar R, Harper MJK, Hanahan DJ. Occurrence of platelet-activating factor in rabbit spermatozoa. *Arch Biochem Biophys* 1988; 260: 497–502.

14 Angle MJ, Jones MA, McManus LM, Pinckard RN, Harper MJK. Platelet-activating factor in the rabbit uterus during early pregnancy. *J Reprod Fertil* 1988; 83: 711–722.

15 Alecozay AA, Casslen BG, Riehl RM et al. Platelet-activating factor in human luteal phase endometrium. *Biol Reprod* 1989; 41: 578–586.

16 Alexander BM, Van Kirk EA, Murdoch WJ. Secretion of platelet-activating factor by periovulatory follicles. *Life Sci* 1990; 47: 865–868.

17 Stein BA, O'Neill C. Morphometric evidence of changes in the vasculature of the uterine tube of mice induced by the 2-cell embryo on the second day of pregnancy. *J Anat* 1994; 185: 397–403.

Cambridge Books Online © Cambridge University Press, 2009

18 Grayson J, Mendel D. *Physiology of splanchnic circulation.* New York: Edward Arnold Ltd; 1965.
19 Morita M, Tsuruta S, Mori KJ, Mayumi M, Mikawa H. Ketotifen inhibits PAF-induced actin polymerization in a human eosinophilic leukaemia cell line, EoL-1. *Eur Respir* 1990; 3: 1173–1178.
20 O'Neill C, Gidley-Baird AA, Pike IL, Porter RN, Sinosich MJ, Saunders DM. Maternal blood platelet physiology and luteal phase endocrinology as a means of monitoring pre- and post-implantation embryo viability following in-vitro fertilisation. *J IVF ET* 1985; 2: 59–65.
21 O'Neill C, Collier M, Saunders DM. Embryo-derived PAF: Its diagnostic and therapeutic future. *Ann NY Acad Sci* 1988; 541: 398–406.
22 Kojima T, Akagi S, Zeniya Y, Shimizu M, Tomizuka T. Evidence of platelet activation associated with establishment of pregnancy in cows with transferred embryos. *J Reprod Devel* 1996; 42: 225–235.
23 Kojima T, Zeniya Y, Ohshima K. Occurrence of early pregnancy-associated thrombocytopenia in splenectomized rabbits. *J Reprod Dev* 1996; 42: 95–100.
24 Ammit AJ, O'Neill C. Comparison of a radioimmunoassay and bioassay for embryo-derived platelet-activating factor. *Hum Reprod* 1991; 6: 872–878.
25 Collier M, O'Neill C, Ammit AJ, Saunders DM. Measurement of human embryo-derived platelet-activating factor (PAF) using a quantitative bioassay of platelet aggregation. *Hum Reprod* 1990; 5: 323–328.
26 O'Neill C. Activity of platelet-activating factor acetylhydrolase in the mouse uterus during the estrous cycle, throughout the preimplantation phase of pregnancy, and throughout the luteal phase of pseudopregnancy. *Biol Reprod* 1995; 52: 965–971.
27 Ammit AJ, O'Neill C. The role of albumin in the release of platelet-activating factor by mouse preimplantation embryos in vitro. *J Reprod Fertil* 1997; 109: 309–318.
28 Ammit AJ, O'Neill C. Studies of the nature of the binding by albumin of platelet-activating factor released from cells. *J Biol Chem* 1997; 272: 18772–18778.
29 O'Neill C, Gidley-Baird AA, Pike IL, Saunders DM. Use of a bioassay for embryo-derived platelet-activating factor as a means of assessing quality and pregnancy potential of human embryos. *Fertil Steril* 1987; 47: 969–975.
30 Ammit AJ, O'Neill C. Optimization of a method for deactivation of platelet-activating factor: acetylhydrolase in serum for use in in-vitro fetilization culture media. *Hum Reprod* 1997; 12: 785–791.
31 Sugio S, Kashima A, Mochizuki S, Noda M, Kobayashi K. Crystal structure of human albumin at 2.5 angstrom resolution. *Pro Eng* 1999; 12: 439–446.
32 Spinks NR, O'Neill C. Antagonists of embryo-derived platelet activating factor prevent implantation of mouse embryos. *J Reprod Fertil* 1988; 84: 89–98.
33 Acker G, Hecquet F, Etienne A, Braquet P, Mencia-Huerta JM. Role of platelet-activating factor (PAF) in the specific ovoimplantation in the rat: effect of the specific PAF-acether antagonist, BN 52021. *Prostaglandins* 1988; 35: 233–241.
34 Ando M, Suginami H, Matsuura S. Pregnancy suppression by a structurally related antagonist for platelet activating factor, CV-6209, in mice. *Asia–Oceania J Obst Gyn* 1990; 16: 283–290.
35 Ando M, Suginami H, Matsuura S. Pregnancy suppression by a platelet-activating factor antagonist, ONO-6240, in mice. *Asia–Oceania J Obst Gyn* 1990; 16: 169–174.
36 O'Neill C. Platelet-activating factor-antagonists reduce implantation in mice at low doses only. *Reprod Fertil Dev* 1995; 7: 51–57.
37 Galione A, White A, Willmott N, Turner M, Potter BVL, Watson SP. cGMP mobilises intracellular Ca^{2+} in sea urchin eggs by stimulating cyclic ADP-ribose synthesis. *Nature* 1993; 365: 456–458.
38 O'Neill C, Ammit AJ, Korth R, Fleming S, Wells X. The effect of inhibitors of platelet aggregation on the metabolism of platelet-activating factor (PAF) in washed rabbit platelets. *Lipids* 1991; 26: 1011–1014.
39 Hirotsune S, Fleck MW, Gambello MJ et al. Graded reductions of Pafahlbl (Lis1) activity results in neuronal migration defects and early embryonic defects. *Nat Genet* 1998; 19: 333–339.
40 Spinks NR, Ryan JP, O'Neill C. Antagonists of embryo-derived platelet activating factor act by inhibiting the ability of the mouse embryo to implant. *J Reprod Fertil* 1990; 88: 241–248.
41 Morton H, Hegh V, Clunie GJA. Studies of the rosette inhibition test in prenant mice: evidence of immunosuppression? *Proc Roy Soc Lond* 1976; 193: 413–419.
42 Orozco C, Perkins T, Clarke FM. Platelet activating factor induces the expression of early pregnancy factor activity in female mice. *J Reprod Fertil* 1986.
43 Cavanagh AC, Morton H. The purification of early-pregnancy factor to homogeneity from human platelets and identification as chaperonin 10. *Eur J Biochem* 1994; 222: 551–560.
44 Cavanagh AC, Morton H. EPF: embryo detector and molecular chaperone. *Today's Life Sci* 1996; 24–27.
45 Athanasas-Platis S, Morton H, Dunglison GF, Kaye PL. Antibodies to early pregnancy factor retard embryonic development in mice in vivo. *J Reprod Fertil* 1991: 92: 443–451.
46 Cavanagh AC, Rolfe BE, Athanasas-Platis S, Quinn KA, Morton H. Relationship between early pregnancy factor, mouse embryo-conditioned medium and platelet activating factor. *J Reprod Fertil* 1991; 93: 355–365.
47 Tonissen K, Wells J, Cock I, Perkins A, Orozco C, Clarke F. Site-directed mutagenesis of human thioredoxin. Identification of cystein 74 as critical to its funtion in the 'early pregnancy factor' system. *J Biol Chem* 1993; 268: 22485–22489.
48 Clarke FM. Identification of molecules and mechanisms involved in the 'early pregnancy factor' system. *Reprod Fertil Dev* 1992; 4: 423–433.
49 Tiemann U, Neels P, Kuchenmeister U, Walzel H, Spitschak M. Effect of ATP and platelet-activating factor on intracellular calcium concentrations of cultured oviductal cells from cows. *J Reprod Fertil* 1996; 108: 1–9.

Cambridge Books Online © Cambridge University Press, 2009

50 Downing SJ, Chambers EL, Maguiness SD, Watson A, Leese HJ. Effect of infammatory mediators on the electrophysiology of the human oviduct. *Biol Reprod* 1999; 61: 657–664.
51 Velasquez LA, Aguilera JG, Croxatto HB. Possible role of platelet-activating factor in embryonic signalling during oviductal transport in the hamster. *Biol Reprod* 1995; 52: 1302–1306.
52 Asuda K, Satouchi K, Saito K. Platelet-activating factor in normal rat uterus. *Biochem Biophys Res Commun* 1986; 138: 1231–1236.
53 Chami O, Megavand A, Ott TL, Bazer FW, O'Neill C. Platelet-activating factor may act as an endogenous pulse-generator for ovine luteolytic $PGF_{2\alpha}$ release. *Am J Physiol* 1999; 576: E782–793.
54 Smith SK, Kelly RW. Effect of platelet-activating factor on the release of PGF-2α by separated cells of human endometrium. *J Reprod Fertil* 1988; 82: 271–276.
55 Battye KM, O'Neill C, Evans G. Evidence that platelet activating factor suppresses uterine oxytocin-induced 13, 14-dihydro-15-keto-prostaglandin FSα release and phosphatidylinositol hydrolysis in the ewe. *Biol Reprod* 1992; 47: 213–219.
56 Ryan JP, O'Neill C, Wales RG. Oxidative metabolism of energy substrates by preimplantation mouse embryos in the presence of platelet-activating factor. *J Reprod Fertil* 1990; 89: 301–307.
57 Ryan JP, Spinks NR, O'Neill C, Wales RG. Implantation potential and fetal viability of mouse embryos cultured in media supplemented with platelet activating factor. *J Reprod Fertil* 1990; 89: 309–315.
58 Roberts C, O'Neill C, Wright L. Platelet activating factor (PAF) enhances mitosis in preimplantation mouse embryos. *Reprod Fertil Dev* 1993; 5: 271–279.
59 Stoddart NR, Wild AE, Fleming TP. Stimulation of development in vitro by platelet-activating factor receptor ligands released by mouse preimplantation embryos. *J Reprod Fertil* 1996; 108: 47–53.
60 O'Neill C. Evidence for the requirement of autocrine growth factors for development of mouse preimplantation embryos in vitro. *Biol Reprod* 1997; 56: 229–237.
61 O'Neill C. Autocrine mediators are required to act on the embryo by the 2-cell stage to promote normal development and survival of mouse preimplantation embryos in vitro. *Biol Reprod* 1998; 58: 1303–1309.
62 O'Neill C, Ryan JP, Collier M, Saunders DM, Ammit AJ, Pike IL. Supplementation of IVF culture media with platelet activating factor (PAF) increased the pregnancy rate following embryo transfer. *Lancet* 1989; ii: 769–772.
63 Roudebush WE, Mathur S, Butler WJ. Anti-platelet activating factor (PAF) antibody inhibits CFW mouse preimplantation embryo development. *J Assist Reprod Gen* 1994; 11: 414–418.
64 Roudebush WE, LaMarche MD, Levine AS, Jiang H, Butler WJ. Evidence for the presence of the platelet-activating factor receptor in the CFW mouse preimplantation two-cell-stage embryo. *Biol Reprod* 1997; 57: 575–579.
65 Emerson M, Travis AR, Bathgate et al. Characterization and functional significance of calcium transients in the 2-cell mouse embryo induced by an autocrine growth factor. *J Biol Chem* 2000; 275: 21905–21913.

Cambridge Books Online © Cambridge University Press, 2009

59

Platelets in psychiatric and neurological disorders

Brian E Leonard

Pharmacology Department, National University of Ireland, Galway, Ireland

Introduction

The difficulty in studying neurotransmitter function in psychiatric patients is legion, despite the initial optimism that surrounded the studies of amine metabolites and neurotransmitter receptors in the brains of suicide victims. It is now generally accepted that factors such as the contribution of amine metabolites from the spinal cord, and the lack of diagnostic certainty in the case of suicide victims, raises serious difficulties when attempting to define the relationship between changes in neurotransmitter function and the symptoms of a psychiatric or neurological disease.

It is against this background that pharmacologists have become interested in the blood platelet as a peripheral model of the nerve terminal. The platelet has many important features in common with the nerve terminal[1]. Thus (a) both the neuron and the platelet contain mitochondria and dense core vesicles in which transmitters are stored. (b) In both cases, the mitochondria provide energy for intracellular functions and also contain monoamine oxidase that catabolizes any free monoamines. (c) The vesicle acts as a storage site for serotonin in the platelet, and most transmitters in addition to serotonin in the neuron, and the release of the amines following activation of the platelet or neuronal membrane is facilitated by a calcium dependent excitation-excretion coupling mechanism. (d) Both platelets and neurons are of the same ectodermal origin and contain neuron specific enolases that, apart from platelets, are only found in nervous tissue. (e) Both platelets and neurons contain functional neurotransmitter and neuromodulator receptor sites on their outer membranes. (f) Both platelets and neurons contain energy-dependent amine transporter sites on their outer membranes. There are also major differences between the platelet and the neuron. Thus the neuron functions as part of a complex nervous network and is not directly influenced by changes in the blood whereas the platelet has no direct connection with the nervous system, has a relatively short half-life (about 10 days) and is directly influenced by changes in the composition of the blood. In addition, whereas the synthesis and metabolism of the neurotransmitters takes place within the neuron, the platelet does not synthesize serotonin, the only major amine transmitter that is of importance for platelet function. In the platelet, the synthesis of serotonin occurs within the amine precursor, uptake and decarboxylation system (APUD cell) which contains all the relevant enzymes necessary for the synthesis for serotonin from its tryptophan precursor. The APUD cells are of the same embryonic origin as the neurons and platelets and may therefore be considered to form part of the paraneuronal system[1]. Thus, while the platelet may be considered to be a useful peripheral model of the serotonergic neuron, it would be incautious to consider it as a relevant model of all types of neuron in the brain. However, a major advantage in studying the platelet lies in its ease of access in patients.

The purpose of this chapter is to critically consider the value of studies of platelet function in some major psychiatric diseases in particular and to demonstrate how such studies may help to understand their pathological bases and response to treatment. It must be emphasized that a disproportionately large amount of data comes from studies of platelet function in depression.

Physiological changes in platelet function of relevance to neurotransmission in the brain

Platelet aggregation is a crucial step in normal hemostasis. Following stimulation by a variety of physiological stimuli such as adenosine diphosphate, adrenaline, serotonin,

thrombin and collagen, platelets undergo a shape change associated with pseudopodia formation and the initiation of the primary reversible phase of aggregation. After a strong physiological stimulus, secretion of the platelet contents takes place with the release of granule proteins and serotonin and the recruitment of additional platelets. This gives rise to the secondary irreversible phase of aggregation. Studies of the two phases of platelet aggregation under controlled conditions in vitro have been valuable in elucidating the complex mechanisms involved in aggregation. These mechanisms have been reviewed by Kroll and Schafer[2] and Leung and Nachman[3] and will not be considered further here.

Activation of platelet aggregation by biogenic amines occurs following the action of the agonists on alpha adrenoceptors and serotonin type 2 receptors[4]. As a consequence of such changes, it has been possible to apply amine-induced platelet aggregation to assess adrenoceptor and serotonin receptor function. For example, nearly two decades ago, Healy et al.[5] showed that a reduction in serotonin-induced aggregation occurred in a group of untreated endogenously depressed patients, an effect which was absent following effective treatment with either amitriptyline or trazodone. Qualitatively, similar changes were reported in a group of depressed patients treated with the selective serotonin reuptake inhibitor (SSRI) antidepressants sertraline and fluoxetine, and the tetracyclic antidepressant mianserin[6,7]. As the normalization of the serotonin-induced aggregatory response did not occur in those patients who failed to respond to antidepressant treatment, it is concluded that the serotonin-induced aggregatory response is a state marker of depression.

Another approach has been to determine serotonin-induced platelet shape change in the presence and absence of the serotonin 2 receptor antagonist ketanserin[8]. While no differences in the platelet shape change occurred between the depressed patients and their controls, it was found that effective treatment with nortriptyline increased platelet aggregation, while those who failed to respond showed no such change. Thus it may be concluded that antidepressants, irrespective of their presumed mechanisms of action, enhance 5-HT 2 receptor function on the platelet membrane. Brusov and coworkers[9] approached the problem of platelet aggregation in a slightly different way. These investigators determined the EC50 values of serotonin-induced aggregation in a small group of depressed women relative to their untreated controls, both before the start of drug treatment and up to 3 months after the commencement of treatment. They found that serotonin-induced platelet aggregation was reduced in the untreated patients but returned to control values in those who responded to treatment lending further support to the view that in depression the platelet 5 HT2 receptor is subfunctional, but returns to normal after effective treatment.

McAdams and Leonard[10] extended the studies of platelet aggregation by comparing a group of patients with major depression with a small group of patients with schizophrenia and with mania before and following effective therapy. They confirmed that effective treatment reverses the reduction in serotonin-induced platelet aggregation in depressed patients, but also showed that a similar reduction in serotonin responsiveness occurred in both schizophrenia and mania. In addition, it was also shown that the aggregatory response to collagen was significantly reduced in both schizophrenic and manic patients, but not in those who were depressed, and remained reduced even following clinical recovery. Thus, a decrease in collagen-induced platelet aggregation appears to be a trait marker of schizophrenia and mania. The action of collagen in inducing platelet aggregation would appear to involve changes in the polyunsaturated fatty acid composition of the membrane[11], which may suggest that the platelet membrane, and possibly the neuronal membrane, are functionally more rigid to physiological stimuli than is normal.

It may be argued that these changes in the aggregatory response to different stimuli are a result of the direct actions of the drugs used to treat the patients and/or due to the effects of the elevated cortisol concentrations which commonly occur in these conditions. This seems unlikely as unpublished evidence by McAdams and Leonard has shown that neither cortisol nor any of the antidepressants, when incubated with platelets and the aggregating agents in vitro had any influence on the aggregatory response.

From these studies, it may be concluded that studies of the aggregating responses of platelets to serotonin and collagen are of some value in identifying changes which are causally related to the pathology of the affective disorders and schizophrenia. However, the question arises regarding the cause of platelet malfunction. This has been the subject of several investigations beginning with those of McAdams et al.[12], who showed that there was a plasma factor which decreased the aggregatory response to serotonin in depressed patients and that its activity was significantly decreased in those patients following recovery. Other investigators had also implicated a plasma factor(s) as responsible for changes in the transport of ^{3}H-serotonin into platelets from depressed patients[9]. Nugent and colleagues[13] extended the studies on the plasma factor(s) by showing that it was stable to freezing, is present in both male and female depressed patients and is equally effective in inhibiting both the primary and secondary aggregatory response. In addition, the plasma factor was

Cambridge Books Online © Cambridge University Press, 2009

found to inhibit the aggregatory response to ADP, noradrenaline, adrenaline, thrombin, collagen and ristocetin. As all these agonists produce their effects by activating specific membrane associated receptors, it would appear that the plasma factor(s) acts on secondary or tertiary messengers within the platelet. Evidence in support of this hypothesis was provided by studies showing that high concentrations of the aggregating agents failed to reverse the inhibitory effects of the plasma factor[13]. It was also shown that the plasma factor(s) inhibited the aggegatory response to sodium fluoride (which activates the guanine nucleotide binding protein system within the platelet[14]), the calcium ionophore A[15] (which stimulates aggregation by increasing the influx of calcium into the platelet[16]) and the phorbol ester PMA (which mediates aggregation by activating protein kinase C[17]).

The identity of the plasma factor(s) is presently unknown, but all the experimental evidence suggests that its action may be at, or beyond, protein kinase C and within the platelet. Furthermore, preliminary studies suggest that the factor is absent from patients with obsessive compulsive disorder, panic disorder, schizophrenia or manic depression[18].

Role of calcium in platelet aggregation

A number of studies have shown that the 5-HT 2A receptor is dysfunctional in mood disorders. For example, the density of these receptors is increased in postmortem brain from depressed patients and suicide victims (for review, see Kusumi and Koyama[19]). In addition, there is evidence that platelets from depressed patients have an increased density of 5-HT 2A receptors which normalize when the patients respond to treatment[6,7,20]. Measurement of serotonin-induced intraplatelet calcium mobilization has also indicated that the 5-HT 2A receptor function is enhanced in unmedicated patients with bipolar disorder or in those with major depression[19,21]. However, unlike the changes in 5-HT 2A density, it appears that the altered calcium response in the affective disorders is a trait-dependent phenomenon. This has been confirmed by Yamawaki et al.[22] and by Berk et al.[23].

In a recent study of bipolar patients and those with major depression, Kusumi et al.[24] showed that those bipolar patients in the depressed phase of their illness who exhibited an enhanced platelet calcium response to serotonin also demonstrated a good clinical response to mood-stabilizing drugs.

The results of this series of clinical studies suggest that serotonin-induced calcium mobilization in the platelet is defective in patients with affective disorders and that, if this is also reflected in the neuron, may help to explain the changes in central serotonin function in these patients. The changes induced by the activation of the 5-HT 2A receptors on the platelet membrane would not appear to be due to those in the basal calcium concentration or in the calcium availability. Thus Dubovsky et al.[15,25] found no difference in either the baseline platelet calcium concentration or in the platelet-activating factor and thrombin-induced calcium response in a group of patients with major depression.

Changes in platelet ^{3}H-serotonin reuptake

Properties of the serotonin transporter on the platelet membrane

The serotonin transporter on both the platelet and the neuronal membrane show absolute dependence on extracellular sodium, a feature known to be characteristic of the Na^+/K^+ cotransport properties where energy, from mitochondrial ATP, for the inward solute transfer is coupled to the energetically favourable influx of Na^+ down its concentration gradient. It is also evident that extracellular Cl^- is absolutely necessary for the normal functioning of the serotonin transporter in the brain. As the platelet and the neuron are similar both in function and in response to physiological stimuli with respect to the serotonin transporter, studies of the transporter on the platelet membrane have some validity. It should be noted, however, that although the structure of the serotonin transporter on the platelet and neuronal membrane of the rat are identical, there appears to be at least three different variants of the transporter in human tissue[26]. Which of these variants is common to the neuron and platelet in human is uncertain, so caution must be expressed in making assumptions about the relevance of changes in the platelet serotonin transporter to those occurring in the brain until this is clarified.

The transport of serotonin into platelets has similar kinetic characteristics to those reported to occur in neurons[27] and therefore it should be possible to use the information obtained from studies of the ^{3}H-serotonin transport into platelets not only to investigate possible changes in serotonergic function in psychiatric and neurological diseases but also to assess how psychotropic drugs may modulate serotonin transport. The most extensive literature on serotonin reuptake into platelets relates to patients with major depression. The first report, by Tuomisto and Tukiainen[28], showed that the serotonin

Cambridge Books Online © Cambridge University Press, 2009

Table 59.1. Effect of antidepressant treatments on the V_{max} of ^{3}H-serotonin reuptake into platelets from depressed patients

Drug	Effect	Reference
Tricyclic antidepressants	Non-reversal of reduced V_{max}	30
Mianserin	Reversed reduced V_{max}	118
Imipramine	Reversed reduced V_{max}	33
Amoxepine	Non-reversal of reduced V_{max}	33
Nortriptyline	Reversed reduced V_{max}	120
Amitriptyline and Trazodone	Reversed reduced V_{max}	5
Lithium	Reversed reduced V_{max}	118
Dothiepin,mianserin and ECT	Reversed reduced V_{max}	74,75
Nomifensin	Reversed reduced V_{max}	34
Sertraline	Reversed reduced V_{max}	6
Peptide analogue with antidepressant effects (INN 00835)	Reversed reduced V_{max}	119

reuptake was decreased in depressed patients. This was confirmed by many other investigators[5,6,29–34]. However, some studies have failed to report an abnormality in platelet serotonin reuptake in depressed patients[35–38] and therefore it has been claimed that a reduced serotonin transport into platelets from depressed patients could be a valuable biological marker of depression[39]. Table 59.1 summarizes the effects of antidepressant treatments on 3H-serotonin reuptake in depressed patients. The majority of these studies indicate that effective drug treatment results in the normalization of serotonin transport suggesting that this is a state marker of depression.

Despite the largely confirmatory evidence that the V_{max} for ^{3}H-serotonin reuptake is a state marker of depression, there is also some preliminary evidence that a similar change occurs in some patients suffering from migraine, hypertension, asthma and myeloproliferative disorders[40]. Patients with panic disorder have also been reported to have a reduced platelet serotonin reuptake, at least in some studies[6,41] but not in others[17,42]. There is also experimental evidence that chronic alcohol administration results in a decreased V_{max} for serotonin reuptake into platelets from rats[43], an effect that was reversed by the chronic administration of omega 6 fatty acids.

The cause of the decreased V_{max} values for serotonin reuptake in untreated depressed patients is difficult to explain. There is evidence that the frequency of the serotonin transporter gene long allele is significantly increased in severely depressed patients[44], which suggests that a functional polymorphism in the serotonin transporter may be associated with severe depression and suicide. In addition, there is evidence that the number of serotonin transport sites on platelet membranes from depressed patients is decreased, and the platelet inositol triphosphate concentration increased[45]; the latter returned to control values following the successful treatment of the patients with a SSRI antidepressant.

One plausible possibility is that hypercortisolemia, a frequent occurrence in depression, is primarily responsible for the change in platelet serotonin dynamics[46]. However, more recently Owens and coworkers[47] examined the changes in serotonin dynamics in rats following the induction of a chronic hyperglucocorticoid state. The results of this study showed that a raised plasma glucocorticoid concentration did not directly affect either the reuptake of platelet serotonin or the affinity of ^{3}H-citalopram for transporter on platelets or synaptosomes. It would therefore appear that changes in the concentration of the glucocorticoids is unlikely to play a role in causing the abnormal serotonin transport associated with depression and other disorders. The possibility remains that the factor(s) that inhibit the serotonin induced aggregatory response of platelets might also inhibit serotonin transport. There is preliminary evidence for such an effect[12] but whether this is the same or a different factor(s) is unknown.

Finally, it is possible that the availability of tryptophan for the APUD cells is abnormal in depression[48], which would adversely impact on the vesicular pool of serotonin in the platelets. However, any change in the availability of the serotonin precursor would not appear to be reflected in the platelet serotonin concentration as there is little evidence of any change in the basal concentration in depressed patients[49,50], even though different types of antidepressants, on chronic treatment, were found to significantly decrease platelet serotonin.

Cambridge Books Online © Cambridge University Press, 2009

^{3}H-imipramine and ^{3}H-paroxetine binding sites on the platelet serotonin transporter

The description of high affinity binding sites for ^{3}H-imipramine in rat and human brain[51,52], as well as on platelets[53,54] has helped to focus attention on the role of modulatory sites in the transport of serotonin. In spite of the methodological differences, many studies have reported a low density (B_{max} values) for ^{3}H-imipramine binding on the platelet membrane from drug-free depressed patients[53–57]. Ellis and Salmond[58] performed a meta-analysis of 76 studies in which platelet ^{3}H-imipramine binding was measured in depressed patients and healthy controls. The result of this analysis showed a highly significant decrease in the B_{max} in the depressed patients. More recent studies have also shown that the B_{max} of the imipramine binding returns to control values after the symptoms of depression have remitted[59–62]. However, the influence of medication on imipramine binding sites is less clear, even though some investigators have shown that antidepressant treatment and concomitant recovery is associated with the increase in the B_{max} values[57]. The reason for the lack of consensus may be related to the heterogenous populations of depressed patients studied, differing methodology for determining platelet ^{3}H-imipramine binding, seasonal factors and the duration of the drug free status of the patients. Another factor that could influence the B_{max} value relates to the plasma cortisol concentration. Unlike ^{3}H-serotonin reuptake or aggregation, a reduction in the B_{max} of platelet imipramine binding correlates well with the cortisol concentration[63], is low in patients with Cushing's disease[64] and in depressed patients who are dexamethasone non-suppressors of cortisol[65]. Thus studies of the ^{3}H-imipramine binding sites on the platelet serotonin transporter may act as a useful marker of depression, but it is uncertain whether this is a state or trait marker of the condition.

Paroxetine is a more specific ligand for labelling a single class of binding sites on the serotonin transporter than imipramine[60] and therefore it would be anticipated that studies in which this ligand, rather than imipramine, is used would give clearer evidence of any abnormality in the serotonin transporter. However, in the few studies in which ^{3}H-paroxetine has been used, no differences have been found between depressed patients and their controls[66–68]. More recently, Nelson and coworkers[69] found no difference between patients with major depression and comorbid obsessive compulsive disorders and their controls.

The assumption has been made that the changes in paroxetine, and possibly also imipramine, binding to the serotonin transporter on the platelet membrane reflect similar changes to those occurring on the neuronal membrane. There is experimental evidence to suggest that this may not be the case. Thus Moret and Briley[70] demonstrated that, while central lesions of the serotonergic system markedly reduced the ^{3}H-paroxetine binding to neuronal membranes, the binding to platelet membranes remained unchanged. It would therefore appear that studies of the imipramine and paroxetine binding sites on the platelet membrane are of only very limited value as markers of the disease state.

The role of platelet adrenoceptors in depression

It is well established that platelets are involved in the pathogenesis of vascular damage in atherosclerosis and arterial hypertension. The reactivity of platelets in vivo is influenced by the activity of the sympathoadrenal system, plasma concentrations of atherogenic lipids and changes in the fluid dynamics of the blood[71]. There is evidence that the human platelet membrane contains both alpha 2A adrenoceptors and dopamine receptors; the alpha 2A are the most prominent and probably responsible for the physiological and pathological changes in platelet function associated with the cardiovascular system. Increases in circulating catecholamines, (for example, caused by physical or mental exercise) initiates responses which include platelet aggregation, secretion of serotonin from the storage granules and activation of the arachidonate pathway[71]. There is evidence that genetic polymorphism of the alpha 2A adrenoceptor encoded by chromosome 10 is associated with hypertension and increased adrenaline mediated platelet aggregation in human[72]. Furthermore, in hypertensive patients, it was shown that subthreshold concentrations of adrenaline potentiated thrombin-induced platelet aggregation.

Epidemiological studies indicate that patients with major depression are predisposed to heart disease. Whether such a predisposition is linked to a polymorphism of the alpha 2 adrenoceptor, as in some types of hypertension, is unknown. However, there are numerous studies showing that the density (B_{max}) of alpha 2 receptors on the platelet membrane of depressed patients is increased. Some of the first studies were published by Garcia Seville et al.[73] and by Healy et al.[73–75] and showed that an increase in the density of alpha 2 receptors on the platelet membranes of drug-free depressed patients returned to control values in those patients who recovered following treatment. Similar increases in the alpha 2 receptor density were sub-

Cambridge Books Online © Cambridge University Press, 2009

sequently reported by Takeda et al.[76], Garcia-Sevilla et al.[77], by Werstiuk et al.[78] and by Gurguis et al.[79] However, there are also studies in which the density of these adrenoceptors in depressed patients was either unchanged or even decreased[80–85]. Possible reasons for these differences may lay in the criteria used for the patient selection, differences in the methods used to prepare the platelet membranes and the differences in the specificities of the ligands used to determine the receptor density. For example, partial agonists, such as ^{3}H-clonidine, indicate an increased density of the alpha 2 receptors[73], while no differences were found when alpha 2 antagonists such as ^{3}H-yohimbine or dihydroergocriptine were used[80].

Determination of the density of a receptor on the platelet membrane does not tell the investigator anything about the functional activity of the receptors. It is therefore essential to determine the activity of the second messenger systems that are activated by the adrenoceptors in order to assess possible changes in functional activity. Newman and coworkers[86] determined both the basal adenylate cyclase activity and also the activities of the second messenger systems that were triggered by sodium fluoride, prostaglandin E1 and forskolin in a group of patients with major depression. These investigators reported no differences in either the basal or stimulated activity of adenylate cyclase in those patients treated with clomipramine together with lithium. Similar results were reported by Garcia-Seville et al.[77], who incidentally also reported such changes in the brains of suicide victims[87]. While such studies suggest that the activity of adenylate cyclase is unchanged in depression, it is known that the alpha 2 receptor exists in different conformational states and therefore any functional change in depression may specifically reflect one of these states which differs from that occurring in the non-depressed state. For example, Gurguis and coworkers[79] showed that the alpha 2A receptor can exist in a low and a high conformational state that is regulated by the Gi protein. In a group of patients with major depression, these investigators showed that the effects of imipramine treatment on the clinical response depended on the conformational state of the receptor. Those patients with a high density of alpha 2 receptors in the higher state had a more positive response to the antidepressant treatment.

Another method for assessing the functional activity of alpha 2 adrenoceptors is to determine platelet aggregation in response to specific alpha 2 agonists. Garcia-Seville et al.[77] demonstrated that the aggregatory response to agonists such as UK-14304 was potentiated in depressed patients, indicating a receptor supersensitivity, but normalised after effective treatment with tricyclic antidepressants. It may be speculated that, although adenylate cyclase is unchanged in depression, it is possible that the activity of protein kinase C is defective, or that the alpha 2 receptor-mediated inositol triphosphate is activated[88].

In addition to the alpha 2 adrenoceptor located on the platelet membrane, there is also evidence that beta adrenoceptors play a role in platelet adhesion and aggregation following prothrombin stimulation. Thus platelet beta 2 receptors mediate inhibition of aggregation and adhesion by stimulation of nitric oxide synthase activity, changes which follow the receptor mediated increase in adenylate cyclase activity[89]. It is not known how the density of platelet beta receptors changes in response to treatment, even though there is considerable evidence to show that the density of beta 2 receptors on lymphocyte membranes is increased in the untreated depressed patient and return to control values following effective treatment[5,6,20]. However, there is some preliminary evidence to indicate that the aggregation response to adrenaline of platelets from normal subjects is reduced when incubated with plasma from depressed patients[18,90], suggesting that an inhibitory plasma factor(s) present in untreated depressed patients affect(s) the functioning of beta adrenoceptors on the platelet membrane.

Because of their lack of specificity for the alpha 2 receptor, attention has recently been focused on the imidazoline receptors, which are also located on platelet membranes and to which many of the alpha 2 receptor ligands are now known to bind. Piletz and coworkers[91] studied changes in the I1 imidazoline receptors and alpha 2 adrenoceptors on platelets from depressed patients. Like the alpha 2 receptors, the platelet I1 receptors were increased in untreated depressed patients, but returned to control values following effective treatment. However, whereas there was a correlation between the plasma adrenaline concentration and the platelet alpha 2 receptor density, there was no correlation between the I1 receptor density and the plasma catecholamine concentration. Differences were also found in the responsiveness of these different receptors and their changes in response to antidepressant treatments. Thus the I1 receptors were decreased in response to both serotonin and noradrenaline reuptake inhibitors, whereas the alpha 2 receptors were primarily responsive to noradrenaline reuptake inhibitors. The results of this study stress the importance in independently assessing both the alpha 2 and imidazoline receptors on platelet membranes before drawing conclusions regarding the possible significance of platelet alpha 2 receptor changes in psychiatric states.

Cambridge Books Online © Cambridge University Press, 2009

Changes in platelet function in headache

A change in the blood serotonin concentration is believed to be a major factor in the initiation of headache particularly in patients with migraine. There is evidence that there is an increase in the basal nitric oxide synthase activity in the platelets from migraine sufferers, which is independent of an acute migraine attack. Sarchielli and coworkers[92] therefore investigated the variation in the nitric oxide synthase and cyclic guanosine monophosphate (cGMP) levels in a group of patients subject to a daily migraine headache by assessing the effect of collagen-induced platelet aggregation on these parameters. The results showed that there was a reduction in the collagen aggregatory response, which was coupled to an increase in both nitric oxide and cGMP synthesis, a significant increase in intracytosolic calcium and a reduction in the platelet serotonin, content. These results therefore suggest that migraine headache, which follows the depletion of platelet serotonin is a reflection of the increased platelet nitric oxide synthesis and increased intracytosolic calcium that follows an abnormal platelet response to collagen stimulation. However, other investigators have cast doubt on the relationship between the free blood serotonin concentration and the pain experienced in a migraine attack. Thus Panconesi et al.[93] reported no correlation between the mechanical overdistension of the hand–forearm veins and the whole blood serotonin concentration. It is therefore possible that other platelet factors, such as nitric oxide, may be the primary cause of the pain associated with the venous distension that occurs in migraine. For example, adenosine, which acts as a potent vasodilator and participates in the control of cerebral and meningial blood flow, is a major factor in migraine headache. For this reason, Guien and colleagues[94] assessed the interaction between adenosine and its antagonists on the release of platelet serotonin. They showed that, during a migraine attack, there was a marked increase in the circulating concentration of adenosine which was associated with the pain. Furthermore, activation of the adenosine A2 receptor on the platelet membrane was found to inhibit serotonin reuptake into platelets, an effect which could result in the rapid elimination of platelet serotonin in migraine sufferers. These results raise the possibility that both adenosine and serotonin play crucial roles in migraine and that adenosine antagonists may be an effective complementary treatment of the condition.

Headache may also be associated with allergic factors and therefore the increase in blood histamine, which may rise in allergic and inflammatory conditions, could play a role. There is evidence, for example, that platelets can store and take up histamine from the blood by an energy dependent process, the uptake being blocked by H1 histamine receptor antagonists[95]. Thrombin is known to release histamine from platelet stores and extracellular histamine causes platelet aggregation and serotonin release. This raises the possibility that headache associated with allergies and inflammatory processes may cause pain not only due to its vasoconstrictor action but also by increasing the release of platelet serotonin.

Changes in platelet function in autism, aggression and impulsivity

Although the literature linking an abnormality in platelet serotonin function with these disorders is limited, there are preliminary indications that there may be a link between changes in serotonin transport into platelets which may reflect the symptoms of these disorders. For example, Cook and Leventhal[96] have shown that the platelet serotonin uptake is increased in patients with autism, which may support the finding that SSRI antidepressants are effective in reducing the ritualistic behaviour and aggression in autistic patients. However, in a group of aggressive patients who were not autistic, Maguire et al.[97] reported no correlation between the psychopathology ratings, aggression and hostility scores and the ^{3}H-paroxetine binding sites on the platelet membrane. Similar results have been obtained in studies of aggressive behaviour in schizophrenic patients[97]. Other studies, however, showed that delinquent boys had a significantly reduced B_{max} for serotonin 2A receptors on platelet membranes that appear to correlate with the degree of antisocial behaviour[98]. The results of the latter two studies emphasize the importance in studying several parameters of platelet serotonin function and that ^{3}H-paroxetine binding studies are of little value in attempting to assess peripheral serotonin function in patients with psychiatric or neurological disorders.

Changes in platelet function in bipolar disorder and schizophrenia

While there has been considerable attention paid to changes in platelet function in patients with major depression, few studies to date have attempted to assess such changes in those with mania or schizophrenia. The comparison between mania and schizophrenia is particularly important not only because the acute symptoms of these disorders frequently overlap[99], but also because such patients are commonly treated with neuroleptics, which

Cambridge Books Online © Cambridge University Press, 2009

may indicate that these conditions share a common biochemical pathology.

Meagher and Leonard[100] studied the changes in platelet aggregation in a small group of patients with acute mania and schizophrenia and compared the aggregation responses to serotonin, noradrenaline and adrenaline with appropriate controls. In an attempt to determine whether the changes in platelet aggregation were correlated with the response to treatment, changes in the aggregatory response to serotonin were also studied during the first 15 days of treatment. The results of this investigation indicated that the serotonin-elicited response was significantly enhanced during the acute phase of the illness, but returned to control values following several weeks of treatment. No changes were found in the adrenaline or noradrenaline-induced platelet aggregatory responses nor was there a correlation between the symptoms of mania, as assessed by the Young Mania Scale, and the serotonin-induced aggregation response. However, unlike the results of the study by McAdams and Leonard[12], no change was found in the serotonin-induced aggregation response in schizophrenic patients. One possible explanation for these differences may lie in the fact that, in the latter study, the patients were unmedicated whereas they were on neuroleptics in the study by Meagher and Leonard[100].

Several studies have been made of changes in platelet serotonin transport in patients with mania. Patients with mania have a higher serotonin content and a higher serotonin reuptake rate than control subjects[39,101–104]. Thus it may be concluded that patients with mania may have an increased presynaptic serotonin concentration. In support of this view, Southam and coworkers[105] have shown that the mood-stabilizing agent lamotrigine dose dependently inhibits the serotonin reuptake into platelets and synaptosomes.

Changes in platelet function in patients with anxiety disorders and fibromyalgia

Both clinical and experimental evidence suggests that an abnormality in noradrenergic and serotonergic function play a crucial role in anxiety disorders. Clinically, it has been shown that the SSRI antidepressants, such as clomipramine and fluvoxamine, reduce the incidence of panic attack. The infusion of lactate into susceptible patients consistently initiates a panic attack. Lingjaerde[106] reported that lactate facilitates the transport of serotonin into platelets which suggest that, if this also occurs in the brain, the intersynaptic concentration of serotonin will be lowered, which could lead to a reduction in the inhibitory effect of serotonin on the firing of the locus coeruleus, the main noradrenergic centre in the brain. Such a mechanism may account for the observed changes in both the serotonergic and noradrenergic systems in patients with panic attack. In support of the imbalance between the noradrenergic and serotonergic systems in panic attack, a detailed study of a group of panic disorder patients before and during treatment with tricyclic antidepressants showed that platelet aggregation to noradrenaline was increased, while that of serotonin was decreased, before treatment and remained largely unchanged following effective treatment[41]. The rate of serotonin transport (V_{max}) was also shown to be decreased, while the density of the serotonin 2A receptors on the platelet membranes was increased in the panic disorder patients irrespective of the response to drug treatment. In a follow-up study of some of the patients conducted some 5 to 6 years later, it was found that the platelet serotonin reuptake was still significantly reduced despite the apparent recovery of the patients[107]. Platelet responses to a number of different aggregatory agents was unchanged relative to the controls. There are qualitatively significant differences between patients with panic disorder and those with depression in that most of the abnormal parameters of platelet function return to control values in those with depression (i.e. act as state markers) whereas many remain abnormal in those with panic disorder (i.e. act as trait markers). However, it should be added that not all investigators have found these changes in patients with panic disorder. Thus some[108] have found no change in ^{3}H-serotonin reuptake into platelets, while others have reported an increase in the serotonin uptake[109]. Similarly, some investigators have shown an increase in the density of alpha-2 adrenoceptors on platelet membranes[6] while others[110] reported no change.

Of the other anxiety disorders in which platelet function has been investigated, there is evidence that the ^{3}H-imipramine binding sites on the serotonin transporter are decreased in patients with obsessive compulsive disorder, the decrease correlating with the severity of the clinical symptoms[111]. Effective treatment of these patients with a SSRI antidepressant largely reversed this abnormality.

Although fibromyalgia cannot be classified as either an anxiety or as an affective disorder, some of the symptoms of the condition do overlap with these disorders. It is therefore of interest to find that the density of the alpha 2 adrenoceptor ligand ^{3}H-rauwulscine on platelet membranes was significantly higher in fibromyalgia than in the control subjects and that the administration of the SSRI sertraline did not reverse these changes. If the results of these platelet studies can be extrapolated to the brain of the fibromyalgia patient, they do suggest that there is a

Cambridge Books Online © Cambridge University Press, 2009

supersensitivity of the inhibitory presynaptic adrenoceptors that may contribute to the general malaise and dysphoria which characterize these patients.

Changes in platelet serotonin transport have also been investigated in women with premenstrual syndrome by Steege and colleagues[112]. It was reported that reduced ^{3}H-imipramine binding occurred in these patients; the decrease in the binding did not correlate with the Beck Depression Inventory Scores, however. Thus changes in serotonergic function may contribute to premenstrual dysphoric symptoms.

Platelet changes in alcoholism

There is experimental evidence to show that the reuptake of ^{3}H-serotonin into platelets is significantly reduced when rats are fed an alcohol rich diet for a period of 21 days[43]. This adverse effect of alcohol on the platelet membrane is reversed by the concurrent administration of a polyunsaturated fatty acid-rich diet, which counteracted the depletion of these fatty acids in the platelet membranes. By contrast, several clinical studies have reported that the platelet ^{3}H-serotonin uptake in alcoholics was higher in alcoholics than in their controls[113], a result which was supported by the later findings of Ernouf et al.[114] and Faraj et al.[115] More recently, Javours and coworkers[116] studied changes in ^{3}H-serotonin reuptake in a group of alcohol-dependent male subjects in which those patients in the early onset category (i.e. they began heavy drinking under the age of 25) were compared with those in the late onset category (i.e. who began heavy drinking after the age of 25). The results indicated that the ^{3}H-serotonin reuptake was higher in the early onset alcoholics than in the late category group, or in the respective controls. Thus, there is a disparity between the experimental and clinical findings with regard to the effects of chronic alcohol consumption on the platelet serotonin transporter. With regard to the other platelet parameters of serotonin function, no change has been reported in the serotonin 2A receptor-stimulated calcium response in platelets in alcoholic men[117].

Conclusions

Much of our understanding of the role of serotonin in psychiatric and neurological disorders is based on peripheral marker studies of serotonin function. As illustrated in this chapter, most of these studies have utilized the platelet as a model of the serotonergic neuron and have concentrated on determining changes in activity of the serotonin transporter, changes in the density and responsiveness of the serotonin 2A receptor and the alpha 2 adrenoceptor on the platelet membrane, changes in the ^{3}H-imipramine binding sites on the serotonin transporter and, finally, changes in the intracellular calcium response of the platelet to serotonin.

This chapter has summarized the results mainly of clinical studies illustrating the importance of the platelet in obtaining an insight into the changes in serotonergic, and to some extent noradrenergic, function in major depression, schizophrenia, mania, the anxiety disorders and also preliminary evidence citing disorders of platelet function in patients with headache, alcoholism and autism. Evidence was also presented that some of these changes in platelet function could be due to plasma factor(s) that, in the case of patients with major depression, appear to be state markers of the condition.

Thus studies of platelet function have been invaluable in enabling an insight to be gained into the role of serotonin in a number of psychiatric and neurological disorders.

References

1 Pletscher A, Affolter H, Cesura M, Erne E, Muller K. Blood platelets as models for neurons: similarities of the serotonin systems. In Schlossberger HG, Kochen W, Linzer B, Steinhart H, eds. *Progress in tryptophan and serotonin research*. Berlin: W De Gruyter & Co; 1984: 231–239.
2 Kroll M, Schafer AI. Biochemical mechanisms of platelet activation. *J Am Soc Hematol* 1989; 74: 1181–1195.
3 Leung L, Nachman R. Molecular mechanisms of platelet aggregation. *Ann Rev Med* 1986; 37: 179–186.
4 Stahl SM, Ciaranello RD, Breger PA. Platelet serotonin in schizophrenia and depression. In Ho BT, ed. *Serotonin in biological Psychiatry*. New York: Raven Press; 1982: 183–198.
5 Healy D, Carney PA, Leonard BE. Monoamine related markers of depression: changes following treatment. *J Psychiat Res* 1983; 17: 251–260.
6 Butler J, Leonard BE. The platelet serotonergic system in depression and following sertraline treatment. *Int Clin Psychopharmacol* 1988; 3: 343–347.
7 Butler J, Leonard BE. Comparison of mianserin and dothiepin on the serotonergic system of depressed patients. *Hum Psychopharmacol* 1990; 5: 369–372.
8 Stahl SM, Hauger R, Rausch JL, Fleishaker JC, Hubbell-Alberts E. Down regulation of serotonin receptor subtypes by nortriptyline and adinazolam in major depressive disorder; neuroendocrine and platelet markers. *Clin Neuropharmacol* 1993; 16 (Suppl. 3): s19–s31.
9 Brusov OS, Beliavev B, Katasonov AB, Zlobina GP, Factor MI, Lideman RR. Does platelet serotonin receptor supersensitiv-

Cambridge Books Online © Cambridge University Press, 2009

ity accompany endogenous depression? *Biol Psychiat* 1989; 25: 375–381.
10 McAdams C, Leonard BE. Changes in platelet aggregatory responses to collagen and serotonin in depressed, schizophrenic and manic patients. *Int Clin Psychopharmacol* 1992; 7: 81–85.
11 McGowan EB, Detwiler TC. Modified platelet responses to thrombin. *J Biol Chem* 1986; 261: 739–746.
12 McAdams C, Colohan FJ, Brophy J, Leonard BE. Alteration by a plasma factor of platelet aggregation and serotonin uptake in depression. *Biol Psychiat* 1992; 32: 296–298.
13 Nugent DF, Dinan TG, Leonard BE. Further characterisation of the inhibition of platelet aggregation by a factor(s) in unmedicated unipolar depressed patients. *J Affect Dis* 1995; 33: 227–231.
14 Kienast J, Arnout J, Pflieger G, Deckmyn H, Hoet B, Vermylen A. Sodium fluoride mimics effects of both agonists and antagonists on intact human platelets by simultaneous modulation of phospholipase C and adenylate cyclase activity. *J Blood* 1987; 69: 859–866.
15 Dubrovsky SL, Christiano J, Daniell LC et al. Increased platelet intracellular calcium concentration in patients with bipolar affective disorder. *Arch Gen Psychiat* 1989; 46: 632–638.
16 Kinlough-Rathbone RL, Packham MA, Reimers HJ, Cazenave JP, Mustard JF. Mechanisms of platelet shape change, aggregation and release induced by collagen, thrombin or A23, 187. *J Lab Clin Med* 1977; 90: 707–719.
17 MacIntyre DE, McNicol A, Drummond AH. Tumour-promoting phorbol esters inhibit agonist-induced phosphatidate formation and calcium influx in human platelets. *FEBS Lett* 1985; 180: 160–164.
18 Nugent DF, Dinan TG, Leonard BE. Alteration by a plasma factor(s) of platelet aggregation in unmedicated unipolar depressed patients. *J Affect Dis* 1994; 31: 61–66.
19 Kusumi I, Koyama T. Serotonin 2A receptor function in affective disorders. In Osawa H, Saito T, Takahata N, eds. *Signal transduction in affective disorders.* Tokyo: Springer Verlag; 1998: 21–34.
20 Healy D, O'Halloran A, Carney PA, Leonard BE. Peripheral adrenoceptors and serotonin receptors in depression: changes associated with trazodone and amitriptyline. *J Affect Dis* 1985; 9: 285–296.
21 Kusumi I, Koyama T, Yamashita I. Effect of various factors on serotonin induced calcium response in human platelets. *Life Sci* 1991; 48: 2405–2412.
22 Yamawaki S, Kagaya A, Okamoto Y, Shimizu M, Nishida A, Uchita M. Enhanced calcium response to serotonin in platelets from patients with affective disorders. *J Psychiat Neurosci* 1996; 21: 321–324.
23 Berk M, Mitchell VS, Plein H. The platelet intracellular calcium response to serotonin in subsyndromal depression. *Int Clin Psychopharmacol* 1998; 13: 107–110.
24 Kusumi I, Suzuki K, Sasaki Y, Kameda K, Koyama T. Treatment response in depressed patients with enhanced calcium mobilisation stimulated by serotonin. *Neuropsychopharmacology* 2000; 23: 690–696.
25 Dubrovsky SL, Lee C, Christiane J, Murphy J. Elevated platelet intracellular calcium concentration in bipolar disorder. *Biol Psychiat* 1991; 29: 441–450.
26 Lesch K-P. Molecular biology, pharmacology and genetics of the serotonin transporter: psychobiological and clinical implications. In Baumgarten HG, Goethert M eds. *Serotoninergic neurons and 5-hydoxytryptamine receptors in the CNS.* Berlin: Springer Verlag; 1997: 671–706.
27 Healy D, Leonard BE. Monoamine transport in depression: kinetics and dynamics. *J Affect Dis* 1987; 12: 91–103.
28 Tuomisto J, Tukiainen E. Decreased uptake of serotonin in platelets from depressed patients. *Nature* 1976; 262: 596–598.
29 Coppen A, Swade C, Wood K. Platelet serotonin accumulation in depressive illness. *Clin Chim Acta* 1978; 87: 165–168.
30 Coppen A, Ghose K, Swade C, Wood K. Effect of mianserin hydrochloride on peripheral uptake mechanisms for noradrenaline and serotonin in man. *Br J Clin Pharmacol* 1978; 5: 13S–17S
31 Aberg-Wistedt A, Jostell KG, Ross SB, Westerlund D. Effects of zimelidine and desipramine on serotonergic and noradrenergic uptake mechanisms in relation to plasma concentrations and to therapeutic effects during treatment of depression. *Psychopharmacology* 1981; 74: 297–305.
32 Scott M, Reading H, Loudon J. Studies on human blood platelets in affective disorders. *Psychopharmacology* 1979; 71: 131–135.
33 Tuomisto J, Tukiainen E, Ahlfors UG. Decreased uptake of serotonin in blood platelets from depressed patients with endogenous depression. *Psychopharmacology* 1979; 65: 141–147.
34 Butler J, Leonard BE. Post-partum depression and the effect of nomifensin treatment. *Int Clin Psychopharmacol* 1986; 1: 244–252.
35 Shaw D, MacSweeney D, Woolcock N, Bevan-Jones A. Uptake and release of 14C-serotonin by blood platelets in affective illness. *J Neurol Neurosurg Psychiat* 1971; 34: 224–225.
36 Kaplan RD, Mann JJ. Altered platelet serotonin uptake kinetics in schizophrenia and depression. *Life Sci* 1982; 31: 583–588.
37 Martensson B, Wagner A, Beck O, Brodin K, Montero D, Asberg M. Effects of clomipramine treatment on CSF monoamine metabolites and platelet ^{3}H-imipramine binding and serotonin uptake and concentration in major depressive disorder. *Acta Psychiat Scand* 1991; 83: 125–133.
38 Goto S, Egashira T, Wada Y, Takayama F, Yamanaka Y. Effect of fluvoxamine on serotonin uptake, paroxetine binding and ketanserin binding sites in the Japanese monkey brain and platelets, in vivo and in vitro. *Jap J Pharmacol* 1996; 71: 291–298.
39 Leonard BE. Changes in platelet serotonergic and adrenergic function in patients with depression, panic attack and mania. In Bunney W, Hippius H eds. *Neuropsychopharmacology* Springer Verlag, Berlin: 1990; 163–171.

Cambridge Books Online © Cambridge University Press, 2009

40 Wood K. Serotonin transport and the mediation of the action of antidepressants: do antidepressant treatments facilitate serotonin transport? In *The biology of depression* Deakin JFW ed. Oxford: Arlen Press; 1986: 90–99.
41 Butler J, O'Halloran A, Leonard BE. The Galway study of panic disorder 2. Changes in some peripheral markers of noradrenergic and serotonergic function in DSM-3R panic disorder. *J Affect Dis* 1992; 26: 89–100.
42 Norman TR, Judd FK, Gregory M et al. Platelet serotonin upake in panic disorder. *J Affect Dis* 1986; 11: 69–72.
43 Corbett R, Menez JF, Leonard BE. The effect of chronic alcohol administration on platelet ^{3}H-serotonin uptake and membrane lipid composition. *Neurochem Int* 1990; 16: 95–98.
44 Du L, Faludi G, Palkovits M et al. Frequency of long allele in serotonin transporter gene is increased in depressed suicide victims. *Biol Psychiat* 1999; 15: 196–201.
45 Alvarez JC, Gluck N, Arnulf I et al. Decreased platelet serotonin transporter sites and increased triphosphate levels in patients with unipolar depression treated with clomipramine and fluoxetine. *Clin Pharmacol Ther* 1999; 66: 617–624.
46 Turner P. Platelet monoamine uptake in depression– a primary or secondary phenomenon. *Hum Psychopharmacol* 1986; 1: 47–48.
47 Owens MJ, Ballenger CA, Knight DL, Nemeroff CB. Platelet serotonin transporter and serotonin binding after chronic hypercortisolemia, citalopram administration or neurotoxin depletion of central nervous system serotonin in the rat. *J Pharmacol Exp Ther* 1996; 278: 1040–1049.
48 Gronier B, Azorin JM, Dassa D, Jeanningros R. Evidence for a defective platelet L-tryptophan transport in depressed patients. *Int Clin Psychopharmacol* 1993; 8: 87–93.
49 Ortiz J, Mariscot C, Alvarez E, Arigas F. Effects of the antiodepressant drug tianeptine on plasma and platelet serotonin in depressive patients and healthy controls. *J Affect Dis* 1993; 29: 227–234.
50 Karege F., Bovier P, Rudolph W, Gaillard TM. Platelet phosphoinositide signalling system: an overstimulation in depression. *Biol Psychiat* 1994; 39: 697–702.
51 Raisman R, Briley MS, Langer SZ. Specific tricyclic antidepressant binding sites in rat brain. *Nature* 1979; 287: 148–150.
52 Langer SZ, Javoy-Agid R, Raisman R, Briley M, Agid Y. Distribution of specific high-affinity binding sites for ^{3}H-imipramine in human brain. *J Neurochem* 1981; 37: 14.
53 Briley MS, Langer SZ, Raisman R, Sechter D, Zarifian E. High affinity binding of ^{3}H-imipramine to human platelets: differences between untreated depressed patients and healthy volunteers. *Science* 1980; 209: 303–305.
54 Paul SM, Rehavi M, Skolnick P, Ballenger JC, Goodwin FK. Depressed patients have decreased binding of tritiated imipramine top platelet serotonin transporters. *Arch Gen Psychiat* 1981; 38: 1315–1317.
55 Asarch KB, Shih JC, Kulcsar A. Decreased ^{3}H-imipramine binding in depressed males and females. *Commun Psychopharmacol* 1980; 4: 425–432.
56 Nemeroff CB, Knight DL, Krishnan KRR et al. Marked reduction in the number of platelet ^{3}H-imipramine binding sites in geriatric depression. *Arch Gen Psychiat* 1988; 45: 919–923.
57 Freeman AM, Stankovic SMI, Bradley RJ, Zang GZ, Libb JW, Nemeroff CB. Tritiated platelet imipramine binding and treatment response in depressed outpatients. *Depression* 1993; 1: 20–23.
58 Ellis PM, Salmond C. Is platelet imipramine binding reduced in depression? A meta-analysis. *Biol Psychiat* 1994; 36: 292–299.
59 Hrdina PD. Imipramine binding sites in brain and platelets: role in affective disorders. *Int J Clin Pharmacol Res* 1989; 9: 119–122.
60 Mellerup ET, Plenge P, Engelsoft M. High affinity binding of ^{3}H-paroxetine and 3H-imipramine to human platelet membranes. *Eur J Pharmacol* 1983; 96: 303–309.
61 Gross-Isseroff R, Beigon A, Voet H, Wezman A. The suicide brain: a review of postmortem receptor/transporter binding studies. *Neurosci Biobehav Revs* 1998; 22: 653–661.
62 Mann JJ. Role of the serotonergic system in the pathogenesis of major depression and suicidal behavior. *Neuropsychopharmacology* 1999; 21: 99S–105S.
63 Paul SM. Serotonin reuptake sites in platelets and human brain: clinical implications. Abstract: World Congress Psychiatry, Regional Meeting, Copenhagen, 1986.
64 Bloomfield JG, Elliott JM, Evans DG. Platelet ^{3}H-imipramine and ^{3}H-yohimbine binding are reduced in Cushing's disease. *Br J Pharmacol* 1986; 86: 432P.
65 Katona CLE, Theodorou AE, Davies SL et al. Platelet binding and neuroendocrine responses in depression. In Deakin JFW ed. *The biology of depression.* Oxford: Arlen Press; 1986: 121–136.
66 Lawrence KM, Katona CLE, AbouSaleh T et al. Platelet serotonin uptake sites labelled with ^{3}H-paroxetine in controls and depressed patients before and after treatment with fluoxetine and lofepramine. *Psychopharmacology* 1994; 115: 261–264.
67 Nankai M, Yamada S, Yoshimoto S et al. Platelet ^{3}H paroxetine binding in control subjects and depressed patients: relationship to serotonin uptake and age. *Psychiat Res* 1994; 51: 147–155.
68 D'Haenen H, DeWaele M, Leysen JE. Platelet ^{3}H-paroxetine binding in depressed patients. *Psychiat Res* 1988; 26: 11–17.
69 Nelson EC, Sheline YI Bardgett ME, Jackson JL, Csernansky JG. Platelet paroxetine binding in major depressive disorder with and without co morbid obsessive-compulsive disorder. *Psychiat Res* 1995; 58: 117–125.
70 Moret C, Briley M. Platelet ^{3}H-paroxetine binding to the serotonin transporter is sensitive to changes in central serotonergic innervation in the rat. *Psychiat Res* 1991; 38: 97–104.
71 Anfossi G, Trovati M. Role of catecholamines in platelet function: pathophysiological changes of clinical significance. *Eur J Clin Invest* 1996; 26: 353–370.
72 Spalding A, Vaitkevicins H, Dill S, MacKenzie S, Schmaier A, Lock P. Mechanism of epinephrine-induced platelet aggregation. *Hypertension* 1998; 31: 603–607.
73 Garcia-Sevilla JA, Zis A, Hollingsworth P, Greden JF, Smith CB.

Cambridge Books Online © Cambridge University Press, 2009

Platelet alpha 2 adrenergic receptors in major depressive disorders. *Arch Gen Psychiat* 1981; 38: 1327–1333.
74 Healy D, O'Halloran A, Carney PA, Leonard BE. Variations in platelet 5-hydroxytryptamine uptake in control and depressed populations. *J Psychiat Res* 1986; 20: 345–353.
75 Healy D, O'Halloran A, Leonard BE. Increases in platelet serotonin uptake rates following treatment with uptake inhibiting drugs. *Int Clin Pharmacol* 1986; 1: 332–339.
76 Takeda T, Harada T, Otsuki S. Platelet ^{3}H-clonidine and ^{3}H-imipramine binding and plasma cortisol in depression. *Biol Psychiat* 1989; 26: 52–60.
77 Garcia-Seville JA, Padro D, Giralt MT, Guiman J, Areso P. Alpha 2 adrenoceptor mediated inhibition of platelet adenylate cyclase and induction of aggregation in major depression. *Arch Gen Psychiat* 1990; 47: 125–132.
78 Werstiuk ES, Cotte M, Griffith L, Shannon H, Steiner M. Effects of electroconvulsive therapy on peripheral adrenoceptors, plasma noradrenaline, MHPG and cortisol in depressed patients. *Br J Psychiat* 1996; 169: 758–765.
79 Gurguis GN, Vo SP, Griffith JM, Rush AJ. Platelet alpha 2A adrenoceptor function in major depression: coupling effects of imipramine and relationship to treatment. *Psychiat Res* 1999; 89: 73–95.
80 Piletz JE, Sletten K. Noradrenergic imidazoline binding sites on human platelets. *J Pharmacol Exp Ther* 1993; 267: 1403–1502.
81 Daiguji M, Meltzer HY, U'Prichard DC. Human platelet alpha 2 adrenergic receptors: labelling with ^{3}H-yohimbine, a selective antagonist ligand. *Life Sci* 1981; 28: 2705–2717.
82 Braddock LE, Cowen PJ, Elliott JM. Binding of yohimbine and imipramine to platelets in depressive illness. *Psychol Med* 1986; 16: 765–773.
83 Katona CLE, Theodoru AE, Davies SL et al. 3H-yohimbine binding to platelet alpha-2 adrenoceptors in depression. *J Affect Dis* 1989; 17: 219–228.
84 Maes M, Van Gastel A, Delmeire L, Meltzer HY. Decreased platelet alpha-2 adrenoceptor density in major depression: effects of tricyclic antidepressants and fluoxetine. *Biol Psychiat* 1999; 45: 278–284.
85 Maes M, Libbrecht I, Delmeire L, Lin A, De Clerck L, Scharpe SJ. Changes in platelet alpha-2 adrenoceptors in fibromyalgia: treatment with antidepressants. *Neuropsychobiology* 1999; 40: 129–133.
86 Newman ME, Lerer B, Lichtenberg P, Shapira B. Platelet adenylate cyclase activity in depression and after clomipramine and lithium treatment: relation to serotonergic function. *Psychopharmacology* 1992; 109: 231–234.
87 Garcia-Seville JA, Escriba PV, Ozaita A et al. Up-regulation of immunolabeled alpha-2A adrenoceptor, G1 coupling proteins and regulatory receptor kinases in the prefrontal cortex of depressed suicides. *J Neurochem* 1999; 72: 282–291.
88 Kenlarts IM, van Gorp RM, Feijge MA, Vuist WM, Heemskerk JW. Alpha 2A adrenergic receptor stimulation potentiates calcium in platelets by modulating camp levels. *J Biol Chem* 2000; 275: 1763–1772.
89 Queen LR, Xu B, Horinouchi K, Fisher I, Ferro A. Beta 2 adrenoceptors activate nitric oxide synthase in human platelets. *Circ Res* 2000; 87: 39–44.
90 Nugent DF, Leonard BE. Platelet abnormalities in depression. *J Serot Res* 1998; 4: 251–266.
91 Piletz JE, Halaris AE, Chikkala D, Qu Y. Platelet I1 -imidazoline binding sites are decreased by two different antidepressant agents in depressed patients. *J Psychiat Res* 1996; 30: 169–184.
92 Sarchielli P, Albert A, Russo S, Codini M, Panico R, Floidi A. Nitric oxide pathway, calcium and serotonin content in platelets of patients suffering from chronic daily headache. *Cephalalgia* 1999; 19: 810–816.
93 Panconesi A, Curradi C, Leoncinci G, Anselmi B, Franchi G. Painful vein overdistension in migraine patients: no relation to serotonergic parameters. *Cephalalgia* 1996; 16: 37–40.
94 Guien R, Devaux C, Henry H et al. Adenosine and migraine. *Can J Neurol Sci* 1998; 25: 55–58.
95 Masini E, DiBello MG, Raspanti S et al. The role of histamine in platelet aggregation by physiological immunological stimuli. *Inflamm Res* 1998; 47: 211–220.
96 Cook EH, Leventhal BL. The serotonin system in autism. *Curr Opin Pediat* 1996; 8: 348–354.
97 Maguire K, Cheung P, Crowley K, Norman T, Schweitzer I, Burrows GB. Aggressive behaviour and platelet ^{3}H-paroxetine binding in schizophrenia. *Schizophren Res* 1997; 23: 61–67.
98 Pine DS, Wasserman GA, Coplan J et al. Platelet serotonin 2A receptor characteristics and platelet factors for boys at risk for delinquency: a preliminary report. *Am J Psychiat* 1996; 153: 538–544.
99 Andreason NC. Thought, language and communication disorders. *Arch Gen Psychiat* 1979; 36: 1325–1330.
100 Meagher J, Leonard BE. Increased serotonin induced platelet aggregation in mania: lack of change following neuroleptic medication. *Hum Psychopharmacol* 1994; 9: 337–342.
101 Modai I, Zemishlany Z, Jerushalmy Z. Serotonin uptake by blood platelets of unipolar and bipolar depressed patients. *Neuropsychobiology* 1984; 12: 93–95.
102 Zemishlany Z, Munitz H, Rotman A, Wijsenbeek H. Increased uptake of serotonin by platelets from patients with bipolar primary affective disorder-bipolar type. *Psychopharmacology* 1982; 77: 175–178.
103 Meagher JB, O'Halloran A, Carney PA, Leonard BE. Changes in platelet serotonin uptake in mania. *J Affect Dis* 1990; 19: 191–196.
104 Shiah I-S, Ko H-C, Lee J-F, Ru-Band L. Increased platelet serotonin in patients with bipolar disorder. *J Affect Dis* 1999; 52: 101–110.
105 Southam E, Kirkby D, Higgins GA, Hagan RM. Lamotrigine inhibits monoamine uptake in vitro and modulates serotonin uptake in rats. *Eur J Pharmacol* 1998; 358: 19–24.
106 Lingjaerde O. Platelet uptake storage of serotonin. In Essman WB, ed. *Serotonin in health and disease*, Vol. 4. New York: Spectrum; 1997; 139–199.
107 Nugent DF, Mannion L, Leonard BE. The Galway study of Panic Disorder 4: Changes in platelet serotonin uptake in a 5–6 year follow-up study. *J Serot Res* 1996; 4: 259–265.

Cambridge Books Online © Cambridge University Press, 2009

108 Schneider P, Evans L, Ross-Lee A et al. Plasma biogenic amine levels in agoraphobia with panic attacks. *Pharmacopsychiatry* 1987; 20: 102–104.
109 McIntyre IM, Judd FK, Burrows GD, Norman TR. Serotonin in panic disorder: platelet uptake and concentrations. *Int Clin Psychopharmacol* 1989; 4: 1–6.
110 Nutt DJ, Fraser S. Platelet binding studies in panic disorder. *J Affect Dis* 1987; 12: 7–11.
111 Marazziti D, Pfanner C, Palego L et al. Changes in platelet markers of obsessive-compulsive patients: double-blind trial of fluvoxamine verses clomipramine. *Pharmacopsychiatry* 1997; 30: 245–249.
112 Steege JF, Stout AL, Knight DL, Nemeroff CB. Reduced platelet tritium-labelled imipramine binding sites in women with premenstrual syndrome. *Am J Obstet Gynecol* 1992; 167: 168–172.
113 Daoust M, Lhuinstre JP, Ernouf D, Legrand E, Breton P, Bouely P et al. Ethanol intake and ^{3}H-serotonin uptake. A study in alcoholic patients using platelet ^{3}H-paroxetine binding. *Life Sci* 1991; 48: 1977–1983.
114 Ernou D, Compagnon P, Lothion P, Narcisse G, Bernard GY, Daoust P. Platelet ^{3}H-serotonin uptake in descendants from alcoholic patients: a potential risk factor for alcohol dependence? *Life Sci* 1993; 52: 989–995.
115 Faraj BA, Olkowski ZL, Jackson RT. Prevalence of higher serotonin in lymphocytes of abstinent alcoholics. *Biochem Pharmacol* 1997; 53: 53–57.
116 Javors M, Tiouririne M, Prihoda T. Platelet serotonin uptake is higher in early-onset than late-onset alcoholics. *Alc Alcohol* 2000; 35: 390–393.
117 Reist C, Helmeste D, Katz M, Vu R, Albers L, Tang SW. Serotonin-stimulated intracellular calcium mobilisation in platelets from alcoholic men. *Psychiat Res* 1995; 57: 275–278.
118 Coppen S, Swade C, Wood K. Lithium restores abnormal platelet serotonin transport in patients with affective disorders. *Br J Pharmacol* 1980; 136: 235–238.
119 Kelly JP, Nicolau G, Redmond A et al. The effect of treatment with a new antidepressant on platelet serotonin uptake in depressed patients. *J Clin Psychiat* 2001; in press.
120 Meltzer HY, Arora RC, Baber RC, Tricon BJ. Serotonin uptake by blood platelets of psychiatric patents. *Arch Gen Psychiat* 1981; 38: 1322–1326.

Cambridge Books Online © Cambridge University Press, 2009

60

Platelets in inflammatory bowel disease

Elizabeth Carty[1], David S. Rampton[2] and Carole E. Collins[3]

[1]*Department of Adult and Paediatric Gastroenterology, St Bartholomew's and The Royal London School of Medicine and Dentistry, London, UK*
[2]*Department of Adult and Paediatric Gastroenterology, St Bartholomew's and The Royal London School of Medicine and Dentistry, London, UK*
[3]*West Middlesex University Hospital, Isleworth, Middlesex, UK*

Introduction

The term inflammatory bowel disease (IBD) encompasses two chronic inflammatory disorders of the gastrointestinal tract, ulcerative colitis and Crohn's disease[1]. The aim of this chapter is to review the abnormalities of platelet number, morphology and function which occur in IBD, the possible role of platelets in its pathogenesis, and the relevance which pharmacological modification of platelet function may have to its treatment.

Inflammatory bowel disease

Ulcerative colitis affects only the colon, while Crohn's may involve any part of the bowel from mouth to anus. The incidence of ulcerative colitis is about 10/100 000/year, and of Crohn's disease about half that figure: their combined prevalence approximates 200/100 000 of the population. Both diseases are commonest in the Western world, and their most frequent age of onset is 20–35 years.

Clinical features

Ulcerative colitis and Crohn's disease of the colon most commonly present with bloody diarrhea, while Crohn's disease of the small intestine tends to cause not only diarrhea but also abdominal pain, weight loss, malaise and fever. Both diseases are characterized by a waxing and waning natural history, and may be complicated, in patients with chronic extensive colitis, by the development of colorectal cancer. Ulcerative colitis and Crohn's disease may be associated with extraintestinal manifestations including iriitis, erythema nodosum, pyoderma gangrenosum, joint disease, a spectrum of hepatobiliary disorders and systemic thromboembolic events.

Thromboembolic disease

Since the 1930s, clinical series have reported that the incidence of arterial and venous thromboembolic disease is increased in patients with IBD, lying between 1 and 8% (Table 60.1). Autopsy studies indicate that underdiagnosis in life is common, and that the true incidence of thromboembolic diseases may be higher (7–41%).

Many of the patients described in the various series have more than one risk factor for thromboembolic events: these include recent abdominal surgery, fluid depletion and bed rest. However, several studies have indicated that thromboembolism in IBD is also common in fully ambulant patients whose disease is in clinical remission[7,12–15].

Treatment

Corticosteroids are the most effective medical treatment of active IBD. Aminosalicylates such as sulfasalazine and mesalazine are useful in the maintenance of remission in ulcerative colitis and are moderately useful in active IBD.

Table 60.1. Increased incidence of thromboembolic disease in inflammatory bowel disease

Clinical studies	Number of cases examined	Incidence of thromboembolic disease (%)
Bargen and Barker (1936)[2]	1500	1.2
Jankelson et al. (1942)[3]	145	3.6
Ricketts and Palmer (1946)[4]	206	1.9
Dennis and Karlson (1952)[5]	261	7.5
Edwards and Truelove (1964)[6]	647	6.4
Talbot et al. (1986)[7]	7199	1.3
Webberley et al (1993)[8]	104	7.8
Postmortem Studies		
Bargen and Barker (1936)[2]	43	31
Warren and Sommers (1949)[9]	180	7
Sloan et al. (1950)[10]	99	41
Graef et al. (1966)[11]	100	39

Patients with refractory disease benefit from immunomodulatory agents such as azathioprine, 6-mercaptopurine, cyclosporin and methotrexate. In Crohn's disease antibiotics, a liquid formula diet and anti-TNF alpha antibody (infliximab) may also be useful. Despite these medical measures, surgery to remove the inflamed bowel is required in up to 20% of patients with ulcerative colitis and in 70% of those with Crohn's disease.

Etiopathogenesis

The Etiopathogenesis of both types of IBD is gradually being unravelled. There is a genetic predisposition to both ulcerative colitis and Crohn's disease, while environmental factors proposed in the pathogenesis include changes in gut microflora, smoking, various drugs and stress. In IBD there is an inappropriately excessive and prolonged mucosal inflammatory response. This response is amplified and perpetuated by recruitment of neutrophils, mononuclear cells and other leukocytes from the intestinal vasculature, with consequent release locally of a variety of proinflammatory cytokines (particularly TNF-alpha and interleukin-1 (IL-1)) and mediators (eicosanoids, platelet activating factor (PAF), reactive oxygen metabolites, proteases).

Multifocal mesenteric microinfarction in Crohn's disease

Evidence, mainly from the Royal Free Medical School in London, suggests that mesenteric vascular injury may be an early pathogenic event in Crohn's disease. This group have proposed a pathogenic sequence, perhaps induced by a persisting virus infection, which comprises focal granulomatous arteritis, fibrin deposition and arterial occlusion at the level of the muscularis mucosa, followed by tissue infarction and mucosal ulceration[16–18] (Fig. 60.1).

Platelets in IBD

Evidence suggesting that platelet activity may have a pathogenic role in IBD includes the increased incidence of thromboembolic disease in affected patients (Table 60.1) and the proposal that microvascular intestinal mucosal thrombosis may be an early event in Crohn's disease[16,17].

What are the abnormalities of platelet number, morphology and function reported in IBD?

Platelet number

Active IBD is associated with thrombocytosis, as first reported in 1968[19]. So high were the platelet counts in this initial study that three of the six ulcerative colitis patients described were given busulfan with reduction of platelet counts to normal in two and prolonged thrombocytopenia in the third. Further reports in larger groups of patients showed a close correlation with other markers of IBD activity, such as hypoalbuminemia, and raised ESR[20] and serum orosomucoid concentration[21]. These findings indicated that platelet counts provide a useful indicator of IBD activity.

Acute diarrhea due to IBD may be difficult to distinguish from infective diarrhea using clinical indicators. Platelet count may be a valuable discriminatory tool: 60% of patients with IBD but only 2% of those with infective diarrhea had a platelet count greater than 450×10^9/l in one study[22].

Platelet size and survival

Studies of mean platelet volume assessed by various methods have shown platelets to be smaller in patients with ulcerative colitis and Crohn's disease compared with controls[8,23,24], consistent with the increased platelet count. Decreased survival half time has been reported[8,20]: since the platelet count is raised in IBD, this observation indicates a greatly increased rate of platelet destruction.

Platelet aggregation

The first report of platelet aggregation, using Born's method, indicated an impaired response to adenosine

Cambridge Books Online © Cambridge University Press, 2009

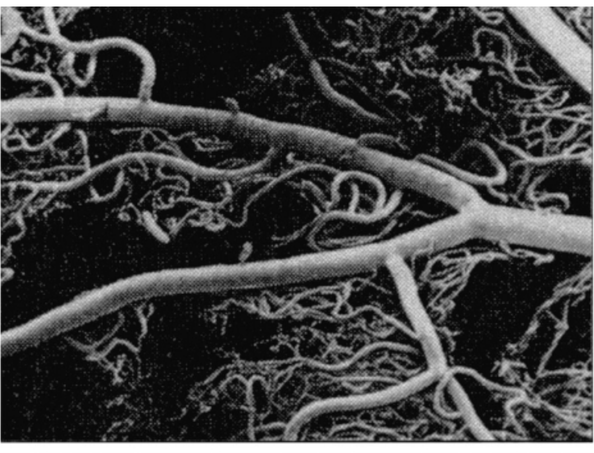


Fig. 60.1. Scanning electron microscopy of resin casts of normal intestine and vascular changes in Crohn's disease. (*a*) Subserosal vessels overlying submucous plexus in normal bowel. (*b*) Crohn's disease showing pronounced stenosis of all vessels subjacent to area of vascular proliferation. (Reproduced with kind permission of *The Lancet* and the author[16].)

diphosphate (ADP), adrenaline and collagen during active IBD which returned to normal with effective treatment[25]. The authors concluded that platelets in active IBD might be 'refractory' due to prolonged low-grade stimulation by ADP released from hemolysed erythrocytes or partially activated platelets; they postulated that their results, paradoxically, provided evidence for increased platelet activation in active IBD.

Subsequent studies have shown that platelet aggregation is enhanced in active IBD, with increased responses occurring to arachidonic acid, ADP, collagen and ristocetin[8,26–28]. Conflicting laboratory results are likely to be explained by differences in methods used for preparing platelet-rich plasma, the most aggregable platelets having been removed from platelet-rich plasma during its preparation from whole blood in the first study[25].

In quiescent IBD, no significant increase in response to ADP was seen in a group of 40 patients[29]. However, in six of these patients, and in none of the healthy controls, spontaneous platelet aggregation in vitro was noted. A later detailed study of platelet-rich plasma taken from 104 patients with active and quiescent disease also reported spontaneous aggregation in vitro in some of the samples[8]. Platelets from these patients, when resuspended in control plasma, continued to exhibit spontaneous aggregation; in contrast, plasma from IBD patients added to control platelets did not induce spontaneous aggregation. Increased aggregation in IBD appeared therefore, to be due to intrinsic platelet abnormalities rather than to the presence of stable proaggregatory compounds in the circulation.

Increased numbers of circulating platelet aggregates in vivo have been detected in patients with active IBD compared to healthy controls and patients with active rheumatoid arthritis not taking NSAIDs[28]. Furthermore, in patients with Crohn's disease undergoing surgery, increased numbers of circulating platelet aggregates were found in mesenteric venous blood in comparison with paired arterial and control mesenteric venous samples[30]: these results suggested that platelet activity was stimulated in the mesenteric microcirculation in Crohn's disease.

Very recent work has shown increased numbers of platelet–leukocyte aggregates in the peripheral blood of patients with IBD, a finding compatible with the hypothesis that platelet activation can influence leukocyte function in this disease[31].

Histological studies demonstrate platelet aggregation in the mesenteric microcirculation in IBD, rectal biopsies from patients with ulcerative colitis showing aggregates plugging mucosal capillaries[32].

Serum proteins and platelet surface markers

Increased plasma platelet factor 4 and beta thromboglobulin have been reported in IBD and, in contrast to results of platelet aggregation studies, did not appear to correlate with disease activity[8,28,33]. More recently, however, positive correlations have been found between these variables and

Cambridge Books Online © Cambridge University Press, 2009

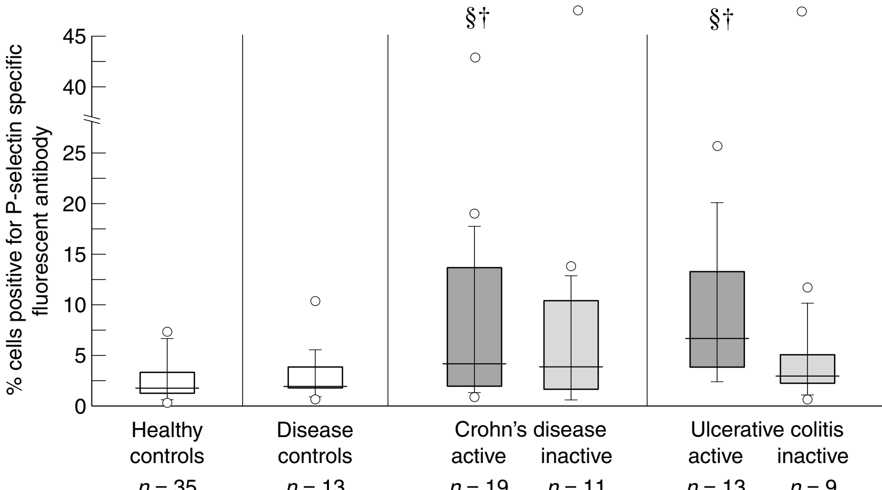


Fig. 60.2. Surface expression of platelet P-selectin in patients with IBD, healthy controls and disease controls (rheumatoid arthritis). For the box and whiskers plots, lines represent medians, boxes interquartile ranges, whiskers 10th–90th centiles and open circles outlying data points. $P<0.05$ vs. healthy controls † $P<0.01$ vs. healthy controls § $P<0.05$ vs. disease controls. (Reproduced with permission from *Gastroenterology*[28].)

indices of activity of Crohn's disease and ulcerative colitis[34]. Differences in results are likely to reflect, primarily, the difficulty of assessing disease activity accurately in IBD with clinical and/or laboratory scoring systems.

Enhanced platelet activation, as indicated by expression of platelet surface P-selectin and GP53, is seen in peripheral blood samples in Crohn's disease and ulcerative colitis independent of disease activity[28,35,36] (Fig. 60.2).

P-selectin expression is greater in finger tip capillary blood than in venous samples in normal individuals. This gradient is exaggerated in patients with Crohn's disease, suggesting that their platelets may be more susceptible to activation in the microcirculation[37].

Possible explanations for abnormal platelet number, morphology and function in IBD

It is not easy to account for the abnormalities of platelet count, size and survival which occur in IBD.

Platelet count is the net result of the balance between production and destruction. IL-3 and IL-6, and thrombopoeitin stimulate thrombopoeisis[38]: plasma IL-6 at least is raised in active Crohn's disease[39].

The data suggesting that, despite being activated, platelets in IBD are small[8,23,24] presents a paradox, since in other disease settings reactivity of platelets correlates with their volume[40–42]. One possible explanation for a reduced platelet volume in the peripheral circulation in IBD could be sequestration or consumption of large activated platelets in the intestinal vasculature. Although such a phenomenon could contribute to reduced platelet survival, attempts to image sequestration in the mesenteric circulation in IBD using radiolabelled platelets have proved inconclusive to date[8].

Release of PAF[43] and thromboxane A_2 (TXA_2)[8] from neutrophils into the mesenteric circulation at the site of mucosal disease could precipitate or amplify platelet activation. A similar effect could be induced by monocytes or neutrophils activated by endotoxin[44], absorption of which is increased in IBD[45].

Basement membrane collagen exposed by endothelial cell damage in the mesenteric circulation is a further possible trigger for increased platelet activation[46]. Increased circulating levels of von Willebrand factor are found in patients with both active and quiescent Crohn's disease demonstrating that endothelial cell damage occurs in this condition[47]. Changes in the endothelium, such as that induced by persistent virus infection, could promote platelet adherence and activation prior to any structural endothelial cell damage[48]. Lastly, exposure of endothelial cells to endotoxin acts as a potent stimulus for platelet aggregation[49].

Pathogenic implications of abnormal platelet function in IBD

Enhanced platelet aggregation and activation may contribute to the pathogenesis of IBD not only through increased release of inflammatory mediators such as PAF, TXA_2, serotonin and reactive oxygen metabolites from

Cambridge Books Online © Cambridge University Press, 2009

platelets themselves, but also through the recruitment, chemotaxis and modulation of the activity of neutrophils, monocytes and other inflammatory cells[50].

Platelet phospholipid membrane provides the template for assembly of the coagulation factors (platelet factor 3) essential for the generation of thrombin and thereby fibrin. If mesenteric microinfarction followed by intravascular fibrin deposition is an important pathogenic mechanism in Crohn's disease[16,17], then platelets, whether activated, aggregated or both, are involved. Their effects in this context are likely to include not only fibrin generation, but also induction of neutrophil accumulation and vasoconstriction as a result of local release of TXA_2 and other soluble mediators.

Platelet aggregates are a potent source of platelet-derived growth factor (PDGF). The mitogenic properties of this factor have been implicated in the pathogenesis of clubbing in cyanotic heart disease[51]. It has been proposed that platelet 'clumps' which, in health, are fragmented in the pulmonary circulation, bypass the pulmonary circulation in cyanotic heart disease and become impacted in the digital circulation. Release of PDGF may stimulate smooth and fibroblast proliferation and thereby account for finger clubbing. The same argument could be applicable in active Crohn's disease[52].

Increased platelet numbers and activation may contribute not only to the pathogenesis of the intestinal lesion in IBD, as indicated above, but also to the increased risk of systemic thromboembolic disease in patients with IBD (Table 60.1).

Therapeutic implications of platelet dysfunction in inflammatory bowel disease

The observations described above provide the rationale for a possible place for antiplatelet agents as a therapeutic strategy in IBD[50].

5-Aminosalicylates

Drugs containing 5-aminosalicylic acid (5ASA) are used in the conventional treatment of IBD and have antiplatelet activity. Sulfasalazine inhibits platelet aggregation in vitro although only at high concentrations (IC_{50} 2 mM)[53]. 5-aminosalicylic acid (1 μM or more) in vitro inhibits platelet activation, while in patients with IBD, taking standard doses of 5-aminosalicylic acid orally inhibits platelet activation in vitro[36] (Fig. 60.3). In patients with IBD, blood and mucosal levels of 5–ASA are 1–10 μM and 1–1000 μM respectively after standard oral dosing[54,55]; thus the inhibition of platelet activation observed in vitro occurs at therapeutically relevant concentrations. However, aminosalicylates have many other pharmacological actions which could explain their efficacy in patients with IBD.

Aspirin and NSAIDS

Aspirin permanently inactivates platelet cyclooxygenase by acetylation and other non-steroidal anti-inflammatory drugs (NSAIDs) reversibly block cyclooxygenase; thus both exert an antiplatelet effect by inhibiting the formation of TXA_2. Not only do these agents have harmful effects on gastrointestinal mucosa[56,57] but NSAIDs have also been associated with reactivation of quiescent IBD[58–60]. It has been suggested that the deleterious effects of NSAIDs in patients with IBD may be due to inhibition of protective prostaglandin production with diversion of arachidonic acid metabolism towards increased synthesis of harmful leukotrienes[57,61]; other proinflammatory mechanisms could also be involved[57]. The possibility that NSAIDs may have a therapeutic role in the prevention of colon cancer suggests that, in the future, these drugs might be used as cancer prevention agents in patients with IBD; careful assessment of their toxicity/benefit ratio would be needed.

Thromboxane synthesis inhibitors

Specific thromboxane synthase inhibitors reduce the formation of TXA_2 in platelets and other cells. They ameliorate colonic inflammation in an animal model of IBD, are anti-inflammatory in studies with inflamed colonic biopsies in vitro, and have shown some benefit in preliminary trials in patients with ulcerative colitis[62–66]. However, a randomized, double-blind, placebo-controlled trial has shown no benefit for the thromboxane synthase inhibitor and receptor blocker, ridogrel, 5 mg daily in patients with active CD[67]. Although it therefore seems unlikely that specifically targeting thromboxanes will be a therapeutically useful approach in IBD, final rejection of the hypothesis that thromboxanes play a pivotal role in its pathogenesis requires a clinical trial using an antithromboxane agent more potent than ridogrel.

Heparin

Heparin has anticoagulant as well as other biological actions, including reduction of leukocyte recruitment[68]. Heparin also inhibits platelet function by antagonizing activation by cathepsin G and inhibiting collagen-induced aggregation[69,70]. While uncontrolled studies have suggested benefits of heparin therapy in patients with active

Cambridge Books Online © Cambridge University Press, 2009

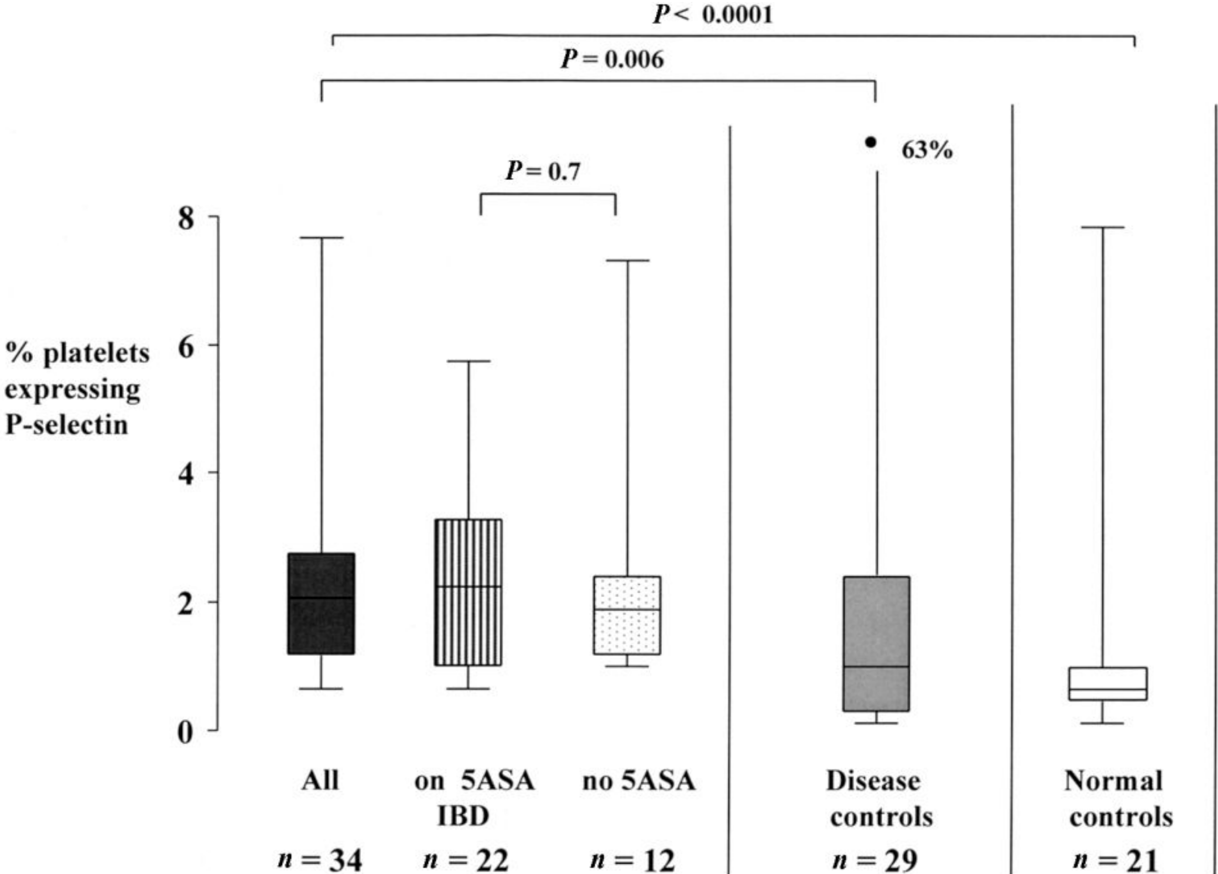


Fig. 60.3. Percentage of platelets expressing P-selectin immediately after venepuncture in patients with IBD, (on and not on 5ASA), disease controls and normal controls. Box and whisker plots are shown, where the box is the interquartile range, the horizontal line the median, and the whiskers show the highest and lowest values. Numbers of patients studied are shown below the *x*-axis. (Reproduced with permission from *Alimentary Pharmacology and Therapeutics*[36].)

UC and CD[71–73], a single controlled trial of heparin monotherapy in UC has had negative results[74]. To date, serious bleeding has been reported only rarely, but results of large-scale randomized controlled trials of heparin therapy in IBD are still awaited.

Antitumour necrosis factor-alpha (TNFα) antibodies

Monoclonal antibodies to TNFα are of proven benefit in patients with otherwise refractory Crohn's disease[75,76] and offer a new therapeutic approach to the treatment of IBD. Studies on markers of coagulation and fibrinolysis in patients with steroid-resistant Crohn's disease given monoclonal TNFα antibodies have shown decreased thrombin generation and endothelial activation markers in association with disease remission.[77] These findings suggest that this therapy could act, in part, by blocking intravascular fibrin deposition in the inflamed gut and thereby reducing any secondary ischemic damage and accelerating healing. However, the place of TNFα at the head of the proinflammatory cascade makes it unlikely that this is its only important mechanism of action in Crohn's disease.

Ticlopidine and clopidogrel

Ticlopidine and its analogue clopidogrel are antagonists of the platelet ADP receptor and are effective in patients with clinical manifestations of atherosclerosis in the coronary and cerebral circulations[78–80]. However, 20% of patients taking ticlopidine develop diarrhea: proposed mechanisms include lymphocytic colitis, small bowel dysmotility and altered prostaglandin production[81,82]. Diarrhea, as well as other serious side effects including thrombotic

Cambridge Books Online © Cambridge University Press, 2009

thrombocytopenic purpura, neutropenia and hepatic dysfunction, make a therapeutic role for this antiplatelet agent in patients with IBD unlikely. Clopidogrel appears to be better tolerated than ticoplidine. However, case reports of thrombotic thrombocytopenic purpura[83] and haemolytic uremic syndrome[84] associated with its use probably preclude assessment of clopidogrel in IBD.

GP IIb/IIIa antibodies

Monoclonal antibodies to the platelet fibrinogen receptor, GPIIb/IIIa, which reduce platelet–platelet and platelet–fibrinogen interactions, are used in patients undergoing coronary artery angioplasty[85]. However, these agents would be unsuitable for evaluation in IBD because of the risk of bleeding associated with their use.

Conclusion

Platelets appear to contribute to the pathogenesis of IBD, and conventional therapy with aminosalicylates inhibits platelet function. However, more specific antiplatelet therapy has not yet been shown to be efficacious in this setting, and it is possible that any benefits of agents potently interfering with platelet function would be outweighed by an increased risk of intestinal mucosal bleeding.

References

1 Rampton DS, Shanahan F. *Fast Facts: Inflammatory Bowel Disease*. Health Press 2000, Oxford, UK.

2 Bargan JA, Barker NW. Extensive arterial and venous thrombosis complicating ulcerative colitis. *Arch Intern Med* 1936; 58: 17–31.

3 Jankelson IR, McClure CW, Sweetsir FN. Chronic ulcerative colitis: II. complications outside the digestive tract. *Rev Gastroenterol* 1942; 9: 99–104.

4 Ricketts WE, Palmer WL. Complications of chronic nonspecific ulcerative colitis. *Gastroenterology* 1946; 7: 55–66.

5 Dennis C, Karlson KE. Surgical measures as supplements to the management of idiopathic ulcerative colitis: cancer, cirrhosis and arthritis as frequent complications. *Surgery* 1952; 32: 892–912.

6 Edwards FC and Truelove SC. The course and prognosis of ulcerative colitis. Part III, complications. *Gut* 1964; 5: 1–15.

7 Talbot RW, Heppell J, Dozois RR, Beart RW, Jr. Vascular complications of inflammatory bowel disease. *Mayo Clin Proc* 1986; 61: 140–145.

8 Webberley MJ, Hart MT, Melikian V. Thromboembolism in inflammatory bowel disease: role of platelets. *Gut* 1993; 34: 247–251.

9 Warren S, Sommers SC. Pathogenesis of ulcerative colitis. *Am J Pathol* 1949; 25: 657–674.

10 Sloan WP, Bargen JA, Gage RP. Life histories of patients with chronic ulcerative colitis: a review of 2000 cases. *Gastroenterology* 1950; 16: 25–38.

11 Graef V, Baggenstoss AH, Sauer WG, Spittell JA Jr. Venous thrombosis occuring in non-specific ulcerative colitis. *Arch Intern Med* 1966; 117: 377–382.

12 Silverstein A, Present DH. Cerebrovascular occlusions in relatively young patients with regional enteritis. *JAMA* 1971; 215: 976–977.

13 Schneiderman JH, Sharpe JA, Sutton DM. Cerebral and retinal vascular complications of inflammatory bowel disease. *Ann Neurol* 1979; 5: 331–337.

14 Novotny DA, Rubin RJ, Slezak FA, Porter JA. Arterial thromboembolic complications of inflammatory bowel disease. Report of three cases. *Dis Colon Rectum* 1992; 35: 193–196.

15 Milandre L, Monges D, Dor V, Juhan-Vague I, Khalil R. Cerebral phlebitis and Crohn disease. *Rev Neurol (Paris)* 1992; 148: 139–144.

16 Wakefield AJ, Sawyerr AM, Dhillon AP et al. Pathogenesis of Crohn's disease: multifocal gastrointestinal infarction. *Lancet* 1989; 2: 1057–1062.

17 Wakefield AJ, Sankey EA, Dhillon AP et al. Granulomatous vasculitis in Crohn's disease. *Gastroenterology* 1991; 100: 1279–1287.

18 Wakefield AJ, Pittilo RM, Sim R et al. Evidence of persistent measles virus infection in Crohn's disease. *J Med Virol* 1993; 39: 345–353.

19 Morowitz DA, Allen LW, Kirsner JB. Thrombocytosis in chronic inflammatory bowel disease. *Ann Intern Med* 1968; 68: 1013–1021.

20 Talstad I, Rootwelt K, Gjone E. Thrombocytosis in ulcerative colitis and Crohn's disease. *Scand J Gastroenterol* 1973; 8: 135–138.

21 Harries AD, Fitzsimons E, Fifield R, Dew MJ, Rhoades J. Platelet count: a simple measure of activity in Crohn's disease. *Br Med J (Clin Res Ed)* 1983; 286: 1476.

22 Harries AD, Beeching NJ, Rogerson SJ, Nye FJ. The platelet count as a simple measure to distinguish inflammatory bowel disease from infective diarrhea. *J Infect* 1991; 22: 247–250.

23 Shah A, Morgan G, Rose JD, Fifield R, Rhodes J. Platelet number and size in relation to serum orosomucoid concentration in Crohn's disease. *Med Lab Sci* 1989; 46: 79–80.

24 Collins CE, Cahill MR, Rampton DS. Paradoxical association between increased platelet activation and reduced platelet volume in Crohn's disease. *Gut* 1993; 34: S63.

25 Lake AM, Stauffer JQ, Stuart MJ. Hemostatic alterations in inflammatory bowel disease: response to therapy. *Am J Dig Dis* 1978; 23: 897–902.

26 Mori K, Watanabe H, Hiwatashi N, Sugai K, Goto Y. Studies on blood coagulation in ulcerative colitis and Crohn's disease. *Tohoku J Exp Med* 1980; 132: 93–101.

Cambridge Books Online © Cambridge University Press, 2009

27 van Wersch JW, Houben P, Rijken J. Platelet count, platelet function, coagulation activity and fibrinolysis in the acute phase of inflammatory bowel disease. *J Clin Chem Clin Biochem* 1990; 28: 513–517.
28 Collins CE, Cahill MR, Newland AC, Rampton DS. Platelets circulate in an activated state in inflammatory bowel disease. *Gastroenterology* 1994; 106: 840–845.
29 Knot E, Ten Cate JW, Leeksma OC, Tytgat GN, Vreeken J. No evidence for a prethrombotic state in stable chronic inflammatory bowel disease. *J Clin Pathol* 1983; 36: 1387–1390.
30 Collins CE, Rampton DS, Rogers J, Williams NS. Platelet aggregation and neutrophil sequestration in the mesoteric circulation in inflammatory bowel disease. *Eur J Gastroenterol Hepatol* 1997; 9: 1213–1217.
31 Irving PM, Azam U, Webb L, Langmead FL, Macey MG, Rampton DS. Leukocyte–platelet aggregation is increased in inflammatory bowel disease. *Gut* 2000; 47(Suppl III): A223.
32 Dhillon AP, Anthony A, Sim R et al. Mucosal capillary thrombi in rectal biopsies. *Histopathology* 1992; 21: 127–133.
33 Simi M, Leardi S, Tebano MT, Castelli M, Costantini FM, Speranza V. Raised plasma concentrations of platelet factor 4 (PF4) in Crohn's disease. *Gut* 1987; 28: 336–338.
34 Vrij AA, Rijken J, van Wersch JW, Stockbrugger RW. Platelet factor 4 and beta-thromboglobulin in inflammatory bowel disease and giant cell arteritis. *Eur J Clin Invest* 2000; 30: 188–194.
35 Schaufelberger HD, Uhr MR, Smith AC, Logan RPH, Gordon-Smith EC, Misiewicz JJ. Platelet activation in inflammatory bowel disease. *Gut* 1993; 34: T196.
36 Carty E, Macey M, Rampton DS. Inhibition of platelet activation by 5-aminosalicylic acid in inflammatory bowel disease. *Aliment Pharmacol Ther* 2000; 14: 1169–1179.
37 Tschoepe D, Schwippert B, Schumacher B, Strauss K, Jackson A, Niederau C. Increased P-selectin (CD62) expression on platelets from capillary whole blood of patients with Crohn's disease. *Gastroenterology* 1993; 104, A793.
38 Ishibashi T, Kimura H, Uchida T, Kariyone S, Friese P, Burstein SA. Human interleukin 6 is a direct promoter of maturation of megakaryocytes in vitro. *Proc Natl Acad Sci USA* 1989; 86: 5953–5957.
39 Mahida YR, Lamming CE, Gallagher A, Hawthorne AB, Hawkey CJ. 5–Aminosalicylic acid is a potent inhibitor of interleukin 1 beta production in organ culture of colonic biopsy specimens from patients with inflammatory bowel disease. *Gut* 1991; 32: 50–54.
40 Thompson CB, Jakubowski JA, Quinn PG, Deykin D, Valeri CR. Platelet size and age determine platelet function independently. *Blood* 1984; 63: 1372–1375.
41 Martin JF, Bath PM, Burr ML. Influence of platelet size on outcome after myocardial infarction. *Lancet* 1991; 338: 1409–1411.
42 Tschoepe D, Roesen P, Esser J et al. Large platelets circulate in an activated state in diabetes mellitus. *Semin Thromb Hemost* 1991; 17: 433–438.
43 Eliakim R, Karmeli F, Razin E, Rachmilewitz D. Role of platelet-activating factor in ulcerative colitis. Enhanced production during active disease and inhibition by sulfasalazine and prednisolone. *Gastroenterology* 1988; 95: 1167–1172.
44 Schwartz BS, Monroe MC. Human platelet aggregation is initiated by peripheral blood mononuclear cells exposed to bacterial lipopolysaccharide in vitro. *J Clin Invest* 1986; 78: 1136–1141.
45 Wellmann W, Fink PC, Benner F, Schmidt FW. Endotoxaemia in active Crohn's disease. Treatment with whole gut irrigation and 5-aminosalicylic acid. *Gut* 1986; 27: 814–820.
46 Cohen I. Role of endothelial injury and platelets in atherogenesis. *Artery* 1979; 5: 237–245.
47 Stevens TR, James JP, Simmonds NJ, McCarthy DA, Laurenson IF, Maddison PJ et al. Circulating von Willebrand factor in inflammatory bowel disease. *Gut* 1992; 33: 502–506.
48 Visser MR, Tracy PB, Vercellotti GM, Goodman JL, White JG, Jacob HS. Enhanced thrombin generation and platelet binding on herpes simplex virus-infected endothelium. *Proc Natl Acad Sci USA* 1988; 85: 8227–8230.
49 Pearson JD. The control of production and release of haemostatic factors in the endothelial cell. *Baillieres Clin Haematol* 1993; 6: 629–651.
50 Collins CE, Rampton DS. Review article: platelets in inflammatory bowel disease – pathogenetic role and therapeutic implications. *Aliment Pharmacol Ther* 1997; 11: 237–247.
51 Dickinson CJ, Martin JF. Megakaryocytes and platelet clumps as the cause of finger clubbing. *Lancet* 1987; 2: 1434–1435.
52 Collins CE, Cahill MR, Rampton DS. Clubbing in Crohn's disease. *BMJ* 1993; 307: 508.
53 Boughton-Smith NK, Hawkey CJ, Rosam AC, Whittle BJ. Sulphasalazine inhibits platelet aggregation and thromboxane formation in human platelets and colon. *Br J Pharmacol* suppl 1986; 88, 391P.
54 Staerk Laursen L, Stokholm M, Bukhave K, Rask-Madsen J, Lauritsen K. Disposition of 5-aminosalicylic acid by olsalazine and three mesalazine preparations in patients with ulcerative colitis: comparison of intraluminal colonic concentrations, serum values, and urinary excretion. *Gut* 1990; 31: 1271–1276.
55 de Vos M, Verdievel H, Schoonjans R, Praet M, Bogaert M, Barbier F. Concentrations of 5-ASA and Ac-5-ASA in human ileocolonic biopsy homogenates after oral 5-ASA preparations. *Gut* 1992; 33: 1338–1342.
56 Bjarnason I, Hayllar J, MacPherson AJ, Russell AS. Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine in humans. *Gastroenterology* 1993; 104: 1832–1847.
57 Wallace JL. Nonsteroidal anti-inflammatory drugs and gastroenteropathy: the second hundred years. *Gastroenterology* 1997; 112: 1000–1016.
58 Rampton DS, Sladen GE. Prostaglandin synthesis inhibitors in ulcerative colitis: flurbiprofen compared with conventional treatment. *Prostaglandins* 1981; 21: 417–425.
59 Walt RP, Hawkey CJ, Langman MJ. Colitis associated with nonsteroidal anti-inflammatory drugs. *Br Med J (Clin Res Ed)* 1984; 288: 238–238.

Cambridge Books Online © Cambridge University Press, 2009

60 Kaufmann HJ, Taubin HL. Nonsteroidal anti-inflammatory drugs activate quiescent inflammatory bowel disease. *Ann Intern Med* 1987; 107: 513–516.

61 Rampton DS, Hawkey CJ. Prostaglandins and ulcerative colitis. *Gut* 1984; 25: 1399–1413.

62 Vilaseca J, Salas A, Guarner F, Rodriguez R, Malagelada JR. Participation of thromboxane and other eicosanoid synthesis in the course of experimental inflammatory colitis. *Gastroenterology* 1990; 98: 269–277.

63 Casellas F, Papo M, Guarner F et al. Effects of thromboxane synthase inhibition on in vivo release of inflammatory mediators in chronic ulcerative colitis. *Eur J Gastroenterol Hepatol* 1995; 7: 221–226.

64 Skandalis N, Rotenberg A, Meuwissen S, De Groot GH, Ouwendijk RJT, Tan TG. Ridogrel for the treatment of mild to moderate ulcerative colitis. *Gastroenterology* 1993; 110: A1016.

65 Wright J, Schenowitz G, Adler G, Schneider H. Ridogrel for the treatment of mild to moderate ulcerative colitis. A placebo controlled trial. *Gut* 1996; 39: A188.

66 Carty E, Macey M, McCartney SA, Rampton DS. Ridogrel, a dual thromboxane synthase inhibitor and receptor antagonist: anti-inflammatory profile in inflammatory bowel disease. *Aliment Pharmacol Ther* 2000; 14: 807–817.

67 Carty E, Rampton DS, Schneider H, Rutgeerts P, Wright JP. Lack of efficacy of ridogret, a thromboxane synthase inhibitor, in a placebo-controlled, double-blind, multi-centre clinical trial in active Crohn's disease. *Aliment Pharmacol Ther* 2000; 15: 1323–1329.

68 Perretti M, Page CP. Heparin and inflammation: a new use for an old GAG? *Gut* 2000; 47: 14–15.

69 Gorog, P.R., Piper, J., Ridler, C.D., Kovacs, I.B. Heparin, both unfractionated and low molecular weight, inhibits physiologically relevant platelet reactivity in native whole blood in vitro. *Thromb Haem* 1991; 65: A849.

70 Cerletti C, Rajtar G, Marchi E, de Gaetano G. Interaction between glycosaminoglycans, platelets, and leukocytes. *Semin Thromb Hemost* 1994; 20: 245–253.

71 Gaffney PR, Doyle CT, Gaffney A, Hogan J, Hayes DP, Annis P. Paradoxical response to heparin in 10 patients with ulcerative colitis. *Am J Gastroenterol* 1995; 90: 220–223.

72 Evans RC, Wong VS, Morris AI, Rhodes JM. Treatment of corticosteroid-resistant ulcerative colitis with heparin – a report of 16 cases. *Aliment Pharmacol Ther* 1997; 11: 1037–1040.

73 Folwaczny C, Wiebecke B, Loeschke K. Unfractioned heparin in the therapy of patients with highly active inflammatory bowel disease. *Am J Gastroenterol* 1999; 94: 1551–1555.

74 Panes J, Esteve M, Cabre E et al. Comparison of heparin and steroids in the treatment of moderate and severe UC. *Gastroenterology* 2000; 119: 903–908.

75 Targan SR, Hanauer SB, van Deventer SJ, Mayer L, Present DH, Braakman et al. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N Engl J Med* 1997; 337: 1029–1035.

76 Rutgeerts P, D'Haens G, Targan S, Vasiliauskas E, Hanauer SB, Present DH et al. Efficacy and safety of retreatment with anti-tumour necrosis factor antibody (Infliximab) to maintain remission in Crohn's disease. *Gastroenterology* 1999; 117: 761–769.

77 Hommes DW, van Dullemen HM, Levi M, van der EA, Woody J, Tytgat GN et al. Beneficial effect of treatment with a monoclonal anti-tumor necrosis factor-alpha antibody on markers of coagulation and fibrinolysis in patients with active Crohn's disease. *Haemostasis* 1997; 27: 269–277.

78 Hass WK, Easton JD, Adams HP, Jr., Pryse-Phillips W, Molony BA, Anderson S et al. A randomized trial comparing ticlopidine hydrochloride with aspirin for the prevention of stroke in high-risk patients. Ticlopidine Aspirin Stroke Study Group. *N Engl J Med* 1989; 321: 501–507.

79 CAPRIE Steering Committee. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. *Lancet* 1996; 348: 1329–1339.

80 More RS, Chauhan A. Antiplatelet rather than anticoagulant therapy with coronary stenting. *Lancet* 1997; 349: 146–147.

81 Berrebi D, Sautet A, Flejou JF, Dauge MC, Peuchmaur M, Potet F. Ticlopidine induced colitis: a histopathological study including apoptosis. *J Clin Pathol* 1998; 51: 280–283.

82 Guedon C, Bruna T, Ducrotte P, Lerebours E, Denis P, Colin R. Severe diarrhea caused by Ticlid associated with disorders of small intestine motility. *Gastroenterol Clin Biol* 1989; 13: 934–937.

83 Bennett CL, Connors JM, Carwile JM, Moake JL, Bell WR, Tarantolo SR et al. Thrombotic thrombocytopenic purpura associated with clopidogrel. *N Engl J Med* 2000; 342: 1773–1777.

84 Moy B, Wang JC, Raffel GD, Marcoux JP. Hemolytic uremic syndrome associated with clopidogrel: a case report. *Arch Intern Med* 2000; 160: 1370–1372.

85 Neumann FJ, Kastrati A, Schmitt C, Blasini R, Hadamitzky M, Mehilli J et al. Effect of glycoprotein IIb/IIIa receptor blockade with abciximab on clinical and angiographic restenosis rate after the placement of coronary stents following acute myocardial infarction. *J Am Coll Cardiol* 2000; 35: 915–921.

Cambridge Books Online © Cambridge University Press, 2009

Cambridge Books Online © Cambridge University Press, 2009

Part IV

Pharmacology

Cambridge Books Online © Cambridge University Press, 2009

Cambridge Books Online © Cambridge University Press, 2009

Aspirin

Carlo Patrono

Department of Pharmacology, University of Rome 'La Sapienza', Rome, Italy

Acetylsalicylic acid was first marketed as aspirin in 1899 and used extensively over the next decades as a prototypic non-steroidal anti-inflammatory drug (NSAID). Following the seminal discoveries made by John Vane and Bengt Samuelsson in the early 1970s on the biochemistry and pharmacology of arachidonic acid metabolism, the molecular mechanism of action of aspirin in inhibiting platelet function was elucidated by the elegant studies of Philip Majerus[1]. The development of whole blood thromboxane (TX)B_2 production as a biochemical endpoint for human studies allowed the characterization of the dose- and time-dependence of the antiplatelet effect of aspirin in the early 1980s. This, in turn, provided the rationale for a new wave of randomized clinical trials employing daily doses 10– to 50-fold lower than used empirically in the past[2]. A large database consisting of over 100 clinical trials of aspirin prophylaxis in a wide range of vascular disorders now provides solid grounds for assessing the balance between benefits and risks in the whole spectrum of atherothrombosis[3,4].

Mechanism of action of aspirin

The best characterized mechanism of action of the drug is related to its capacity to inactivate permanently the cyclooxygenase (COX) activity of prostaglandin H-synthase (PGHS)-1 and -2 (also referred to as COX-1 and COX-2)[5]. These isozymes catalyse the first committed step in prostanoid biosynthesis, i.e., the conversion of arachidonic acid to PGH_2. PGH_2 is the immediate precursor of PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2. COX-1 and COX-2 are homodimers of a ~72 kDa monomeric unit. Within the enzymatic domain, there is a peroxidase catalytic site and a separate, but adjacent, site for COX activity at the apex of a long, hydrophobic channel. There are a number of important differences between COX-1 and COX-2, some of which may contribute to variable inhibitor selectivity[5].

The molecular mechanism of permanent inactivation of COX activity by aspirin is related to blockade of the COX channel as a consequence of acetylation of a strategically located serine residue (Ser529 in the human COX-1, Ser516 in the human COX-2), that prevents access of the substrate to the catalytic site of the enzyme[6]. Because aspirin has a short half-life (15–20 min) in the human circulation and is approximately 50– to 100-fold more potent in inhibiting platelet COX-1 than monocyte COX-2[7], it is ideally suited to act on anucleate platelets, inducing a permanent defect in TXA_2-dependent platelet function. Moreover, since aspirin probably also inactivates COX-1 in relatively mature megakaryocytes, and since only 10% of the platelet pool is replenished each day, once-a-day dosing of aspirin is able to maintain virtually complete inhibition of platelet TXA_2 production[8]. In contrast, inhibition of COX-2-dependent pathophysiologic processes (e.g. hyperalgesia and inflammation) requires larger doses of aspirin (because of lower sensitivity of COX-2 to aspirin) and a much shorter dosing interval (because nucleated cells rapidly resynthesize the enzyme). This results in markedly different benefit/risk profiles of the drug, depending on the clinical indication and its variable dose requirements[2,3].

Pharmacokinetics

Aspirin is rapidly absorbed in the stomach and upper intestine. Peak plasma levels occur 30 to 40 min after aspirin ingestion, and inhibition of platelet function is evident by 1 h. In contrast, it can take up to 3 to 4 h to reach peak plasma levels after administration of enteric-coated aspirin. If only enteric-coated tablets are available, and a rapid effect is required, the tablets should be chewed. The

oral bioavailability of regular aspirin tablets is approximately 40 to 50% over a wide range of doses[9]. A considerably lower bioavailability has been reported for enteric-coated tablets and sustained-release, microencapsulated preparations. Because platelet COX-1 is acetylated in the presystemic circulation, the antiplatelet effect of aspirin is largely independent of systemic bioavailability[9]. Both a controlled-release formulation[10] and a transdermal patch[11] with negligible systemic bioavailability have been developed in an attempt to achieve selective inhibition of platelet TXA_2 production without suppressing systemic PGI_2 synthesis. However, the clinical relevance of preparations relatively selective for the presystemic circulation remains to be established (see below).

The plasma concentration of aspirin decays with a half-life of 15 to 20 min. Despite the rapid clearance of aspirin from the circulation, the platelet-inhibitory effect lasts for the lifespan of the platelet because aspirin irreversibly inactivates platelet COX-1[1]. Aspirin also acetylates the enzyme in megakaryocytes before new platelets are released into the circulation. The mean lifespan of human platelets is approximately 10 days. Therefore, about 10% of circulating platelets are replaced every 24 h, and 5 to 6 days following aspirin ingestion, approximately 50% of the platelets function normally[1,8].

Pharmacodynamics

Effects on TXA_2 and PGI_2

Human platelets and vascular endothelial cells process PGH_2 to produce TXA_2 and prostacyclin (PGI_2), respectively[1]. TXA_2 induces platelet aggregation and vasoconstriction, while PGI_2 inhibits platelet aggregation and induces vasodilation. Aspirin is antithrombotic in a wide range of doses inhibiting TXA_2 and PGI_2[2]. While TXA_2 is largely a COX-1-derived product (mostly from platelets) and thus highly sensitive to aspirin inhibition, vascular PGI_2 can derive from both COX-1 (short-term changes in response to agonist stimulation, e.g. bradykinin; sensitive to transient aspirin inhibition) and COX-2 (long-term changes in response to laminar shear stress and inflammatory cytokines; largely insensitive to aspirin inhibition at conventional antiplatelet doses)[5,12,13]. This may account for the substantial residual COX-2-dependent PGI_2 biosynthesis in vivo at daily doses of aspirin in the range of 30 to 100 mg, despite transient suppression of COX-1-dependent PGI_2 release[10,14]. It is not established that more profound suppression of PGI_2 formation by higher doses of aspirin is sufficient to initiate or predispose to thrombosis. However, studies with mice deficient in the gene encoding the PGI_2 receptor support the importance of this prostanoid in the prevention of arterial thrombosis[15].

Effects of aspirin not related to TXA_2

Aspirin has been reported to have effects on hemostasis that are unrelated to its ability to inactivate platelet COX-1. These include dose-dependent inhibition of platelet function, enhancement of fibrinolysis, and suppression of plasma coagulation. In contrast to the saturable and well-characterized (nanomolar aspirin concentration, rapid time course, physiological conditions, single serine modification) inhibition of COX-1 by aspirin, the putative mechanisms underpinning the 'non-prostaglandin' effects of aspirin on hemostasis are dose dependent and less clearly defined[3].

A subgroup analysis of the Physicians' Health Study, based on *post-hoc* measurements of baseline plasma C-reactive protein (CRP: the prototypic acute phase protein, whose serum levels can increase in response to tissue damage, infection or inflammation) performed in 543 apparently healthy men who subsequently developed MI, stroke or venous thrombosis, and in 543 study participants who did not report vascular complications, has found that the reduction in the risk of a first MI associated with the use of aspirin (325 mg on alternate days) appears to be directly related to the level of CRP, raising the possibility of anti-inflammatory as well as antiplatelet effects of the drug in cardiovascular prophylaxis[16]. As noted above, the anti-inflammatory effects of aspirin and other NSAIDs are largely related to their capacity to inhibit COX-2 activity induced in response to inflammatory cytokines, as these clinical effects can be fully reproduced by highly selective COX-2 inhibitors (coxibs) in patients with rheumatoid arthritis[17]. The dose- and time dependence of the effects of aspirin on nucleated inflammatory cells expressing COX-2 vs anucleated platelets expressing COX-1 are markedly different, thus making an anti-inflammatory effect of the drug at 325 mg every other day pharmacologically implausible, given its very short half-life in the human circulation[3].

Finally, aspirin has been reported to modify the way in which neutrophils and platelets or erythrocytes and platelets interact, to protect endothelial cells from oxidative stress and to improve endothelial dysfunction in atherosclerotic patients. However, neither the molecular mechanism(s) nor the dose dependence of these effects have been clearly established.

Cambridge Books Online © Cambridge University Press, 2009

Aspirin 'Resistance'

Aspirin 'resistance' has been used to describe a number of different phenomena, including the inability of aspirin to: (i) protect individuals from thrombotic complications, (ii) cause a prolongation of the bleeding time, or (iii) produce an anticipated effect on one or more in vitro tests of platelet function[3]. A variable proportion (up to 1/4) of patients with cerebrovascular disease only achieve partial inhibition of platelet aggregation at initial testing, and some (up to 1/3) seem to develop 'resistance' to aspirin over time, even with increasing doses. The results of these long-term studies carried out by Helgason et al.[18–20] are at variance with those of a short-term study of Weksler et al. showing that 40 mg aspirin daily inhibited platelet aggregation and TXA_2 formation as effectively as higher doses of aspirin in patients who had recent cerebral ischemia[21]. Variable platelet responses to aspirin have also been described in patients with peripheral arterial disease and with ischemic heart disease. In the study of Buchanan and Brister, aspirin 'non-responders' were identified on the basis of bleeding time measurements[22]. Approximately 40% of patients undergoing elective coronary artery bypass grafting showed no prolongation of bleeding time in response to aspirin. This was associated with increased platelet adhesion and 12–HETE synthesis[22]. In contrast, repeated measurements of platelet aggregation carried out over 24 months of placebo-controlled treatment by Berglund and Wallentin demonstrated that 100 patients with unstable coronary artery disease randomized to receive 75 mg aspirin daily in the RISC study had consistently reduced platelet aggregation without attenuation during long-term treatment[23].

Several relatively small studies in stroke patients have suggested that aspirin 'resistance' may contribute to treatment 'failure', i.e., recurrent ischemic events while on antiplatelet therapy, and that doses higher than 500 mg may be more effective than lower doses in limiting this phenomenon. The uncontrolled nature and small sample size of these studies makes it difficult to interpret the results. As discussed below, a much larger database failed to substantiate a dose-dependent effect of aspirin in stroke prevention, an effect that one would theoretically expect if aspirin 'resistance' could be overcome, at least in part, by increasing the daily dose of the drug. The apparent discrepancy between the theoretical predictions originating from studies of aspirin 'resistance' and the actual findings of approximately 100 randomized clinical trials of aspirin prophylaxis in high-risk patients[4] can be reconciled by acknowledging the limitations of platelet function studies. Thus, platelet aggregation as measured by conventional methods ex vivo has less than ideal intra- and intersubject variability and displays limited sensitivity to the effect of aspirin, often considered a 'weak' antiplatelet agent based on such measurements. Moreover, the relevance of changes in this index of capacity to the actual occurrence of platelet activation and inhibition in vivo is largely unknown. Similarly, the bleeding time has serious problems of methodologic standardization and is of limited value in predicting hemostatic competence.

In a recent study, Weber et al. reported that circulating platelets from healthy subjects express COX-2 protein and mRNA, and suggested that this may represent a factor in aspirin 'resistance'[24]. This finding has been disputed by several groups. On the other hand, incomplete suppression of 11-dehydro-TXB_2 excretion – a non-invasive index of in vivo TXA_2 biosynthesis – has been observed episodically in some patients with unstable angina treated with i.v. low-dose aspirin, despite greater than 95% suppression of platelet COX-1 activity[25]. COX-2 induction in plaque monocytes/macrophages or activated endothelial cells may contribute to aspirin-insensitive TXA_2 biosynthesis in unstable angina by generating PGH_2 as a substrate for the TX-synthase of the same cell ('constitutive' biosynthesis) or by providing PGH_2 to the TX-synthase of aspirinated platelets ('transcellular' metabolism)[26]. The clinical importance of this phenomenon remains to be established.

The coadministration of aspirin and other NSAIDs can lead to pharmacodynamic interactions between the two, leading to attenuation of the antiplatelet effect of aspirin. Because aspirin's ability to acetylate a critical serine residue at the apex of the cyclooxygenase channel is dependent upon its initial binding to arginine-120, a common docking site for all NSAIDs, the stronger binding affinity of non-aspirin NSAIDs may preclude aspirin from permanently modifying platelet COX-1[6]. A highly selective COX-2 inhibitor, such as rofecoxib, is less likely to interfere with the antiplatelet effect of aspirin than conventional NSAIDs because of its limited interaction with platelet COX-1[17]. Finally, it is theoretically possible that polymorphisms and/or mutations in the COX-1 gene affecting Ser529 may represent the structural basis for aspirin 'resistance' in some patients, although this hypothesis remains to be tested.

Thus, in summary, both the mechanism(s) and clinical relevance of aspirin 'resistance' remain to be established. Until its true nature and prevalence are better defined, no test of platelet function is recommended to assess the antiplatelet effect of aspirin in the individual patient[3].

Cambridge Books Online © Cambridge University Press, 2009

The antithrombotic effects of aspirin

Well-designed randomized trials have shown that aspirin is an effective antithrombotic agent when used in doses ranging between 50 and 100 mg/d and there is a suggestion that it is effective in doses as low as 30 mg/d[2–4]. Aspirin in a dose of 75 mg/d was shown to be effective in reducing the risk of acute myocardial infarction (MI) or death in patients with unstable angina[27] and chronic stable angina[28] as well as in reducing stroke or death in patients with transient cerebral ischemia[29] and the number of postoperative strokes after carotid endarterectomy[30]. In the European Stroke Prevention Study II[31], aspirin, 25 mg bid, was effective in reducing the risks of stroke and stroke or death in patients with prior stroke or transient ischemic attack (TIA). Finally, in a study of 3131 patients after a TIA or minor ischemic stroke, aspirin in a dose of 30 mg/d was compared with a dose of 283 mg/d, and no statistically significant difference was found in the incidence of the combined outcome of vascular death, stroke, or myocardial infarction between the two aspirin regimens[32]. The lowest effective dose of aspirin for these various indications is shown in Table 61.1.

Table 61.1. Vascular disorders for which aspirin has been shown to be effective and minimum effective dose

Disorder	Minimum effective daily dose (mg)
Men at high cardiovascular risk	75
Hypertension	75
Stable angina	75
Unstable angina[a]	75
Acute myocardial infarction	160
Transient ischemic attack and ischemic stroke[a]	50
Severe carotid artery stenosis[a]	75
Acute ischemic stroke[a]	160

Note:
[a] Higher doses have been tested in other trials and not found to confer any greater risk reduction.
Source: Reproduced with permission[3].

The clinical effectiveness of different doses of aspirin has been compared directly in a small number of randomized trials. In the United Kingdom–TIA (UK–TIA) study, no difference in efficacy was found between 300 and 1200 mg/d of aspirin[33]. The Dutch TIA study failed to show a difference between 30 and 283 mg/d of aspirin. The Aspirin and Carotid Endarterectomy trial[34] has recently reported that the risk of stroke, MI or death within 3 months of carotid endarterectomy is significantly lower for patients taking 81 mg or 325 mg aspirin daily than for those taking 650 mg or 1300 mg (6.2% vs. 8.4%; $P = 0.03$). Thus, there is no convincing evidence from randomized studies that have compared different doses of aspirin for a dose dependence of the antithrombotic effect[3,4].

The antithrombotic effects of a range of doses of aspirin have also been compared with an untreated control group in a number of thrombotic vascular disorders. The doses have varied between 50 and 1500 mg/d. Aspirin has been shown to be effective in the following conditions[2–4]: unstable angina in which the incidence of acute MI or death was significantly reduced to a similar degree in four separate studies using daily doses of 75 mg, 325 mg, 650 mg, and 1300 mg; stable angina in which a dose of 75 mg daily reduced the incidence of acute MI or sudden death; aorto-coronary bypass surgery in which the incidence of early occlusion was similarly reduced with daily doses of 100 mg, 325 mg, 975 mg, and 1200 mg; thromboprophylaxis of patients with prosthetic heart valves who also received warfarin in whom the incidence of systemic embolism was reduced with daily doses of 100 mg, 500 mg, and 1500 mg; thromboprophylaxis of patients with arterial venous shunts undergoing long-term hemodialysis in whom a dose of 160 mg/d was shown to be effective; acute MI in which a dose of 162.5 mg/d reduced early (35 day) mortality as well as non-fatal reinfarction and stroke; transient cerebral ischemia in which doses between 50 and 1200 mg/d were effective; and acute ischemic stroke in which doses of 160 to 300 mg/d were effective in reducing early mortality and stroke recurrence.

Although the proportional effects of aspirin therapy on major vascular events (non-fatal MI, non-fatal stroke or vascular death) in these high-risk settings have been characterized as relatively homogeneous ($23 \pm 2\%$ odds reduction) based on a recent overview of all secondary prevention studies[4], individual trial data show substantial heterogeneity ranging from no apparent benefits in patients with peripheral arterial disease to ≥50% risk reductions in patients with unstable coronary syndromes. These findings may reflect the variable importance of TXA_2 as a mechanism amplifying the hemostatic response to plaque destabilization in different clinical settings[3].

Finally, the variable underlying risk of developing a major vascular event if untreated appears to represent the major determinant of the absolute benefit of aspirin prophylaxis in different patient groups, as depicted in Fig. 61.1[3].

Cambridge Books Online © Cambridge University Press, 2009

Table 61.2. 'Primary' prevention trials of aspirin vs. placebo

Trial	Subjects (*n*)	Follow-up yr	Placebo event rate % per yr	Aspirin RR
UK Doctors[36]	Healthy men (5139)	5.8	1.4	1.03
US Physicians[35]	Healthy men (22 071)	5.0	0.7	0.82
TPT[37]	High-risk men (5085)	6.3	1.6	0.83
PPP[38]	High-risk men and women (4495)	3.6	0.8	0.71
HOT[34]	Hypertensive pts (18 790)	3.8	1.1	0.85
SAPAT[36]	Stable angina pts (2035)	4.2	3.7	0.71

Note:
RR = relative risk of non-fatal MI, non-fatal stroke or vascular death.
Source: Reproduced in updated form[3].

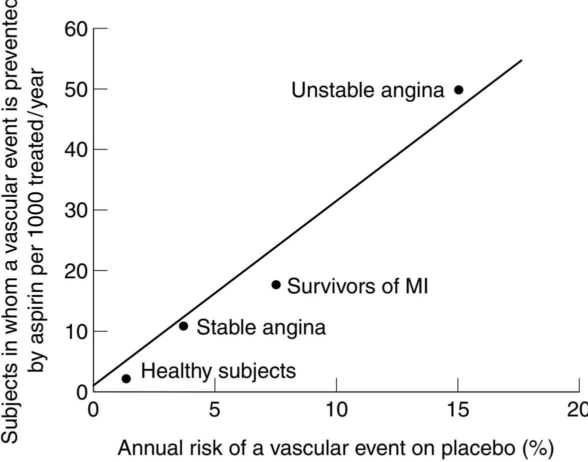


Fig. 61.1. The absolute risk of vascular complications is the major determinant of the absolute benefit of antiplatelet prophylaxis. Data are plotted from placebo-controlled aspirin trials in different clinical settings. For each category of patients, the abscissa denotes the absolute risk of experiencing a major vascular event as recorded in the placebo arm of the trial(s). The absolute benefit of antiplatelet treatment is reported on the ordinate as the number of subjects in whom an important vascular event (non-fatal MI, non-fatal stroke, or vascular death) is actually prevented by treating 1000 subjects with aspirin for 1 year. (Reproduced with permission[3].)

Aspirin and primary prevention

Aspirin has been evaluated in six 'primary' prevention trials in approximately 58 000 persons at variable cardiovascular risk (Table 61.2)[35–40]. In the US Physicians' Health Study (PHS), among 22 071 healthy physicians, an alternate-day regimen of 325 mg of aspirin conferred a statistically significant 44% reduction in risk of first MI[35]. Neither the overall cardiovascular mortality, which was the primary end point of the study, nor the total number of strokes was reduced by long-term aspirin prophylaxis, but there was evidence of a possible increase in hemorrhagic strokes. The British Doctors' trial[36] found no statistically significant effects of aspirin, 500 mg daily, but it had a much smaller sample size than the US trial. An overview of both trials suggested a highly significant 32% reduction in risk of first MI, but the data for stroke and cardiovascular mortality were inconclusive[40]. In terms of absolute benefit, the protective effect of aspirin translated into a major vascular event being avoided in four healthy physicians per 1000 men treated for 5 years. It should be emphasized that this self-selected group of health-conscious male physicians had an absolute risk of developing a major vascular event, if untreated, of <1.0% per year, much lower than the expected rate in the general population.

In the Thrombosis Prevention Trial (TPT)[37], 5499 men aged between 45 and 69 years were recruited from >100 general practices in the United Kingdom because they were considered to be at high risk of ischemic heart disease (in the top 20 to 25% of the risk score distribution). Low-dose aspirin therapy, 75 mg/d, in a controlled-release formulation discussed above, produced a statistically significant 20% relative reduction in the risk of the primary end point of all ischemic heart disease (i.e., coronary death, fatal or non-fatal MI), which was almost entirely due to a 32% reduction in nonfatal events[37]. As in the two previous 'primary' prevention trials, neither the total number of strokes nor the overall cardiovascular mortality was modified by aspirin prophylaxis in TPT. Although the men recruited in TPT were selected for being at high risk of ischemic heart disease, their actual risk of developing a major vascular complication, as assessed in the control group, was considerably lower than expected and only a little more than the rate found in the British Doctors' Trial,

Cambridge Books Online © Cambridge University Press, 2009

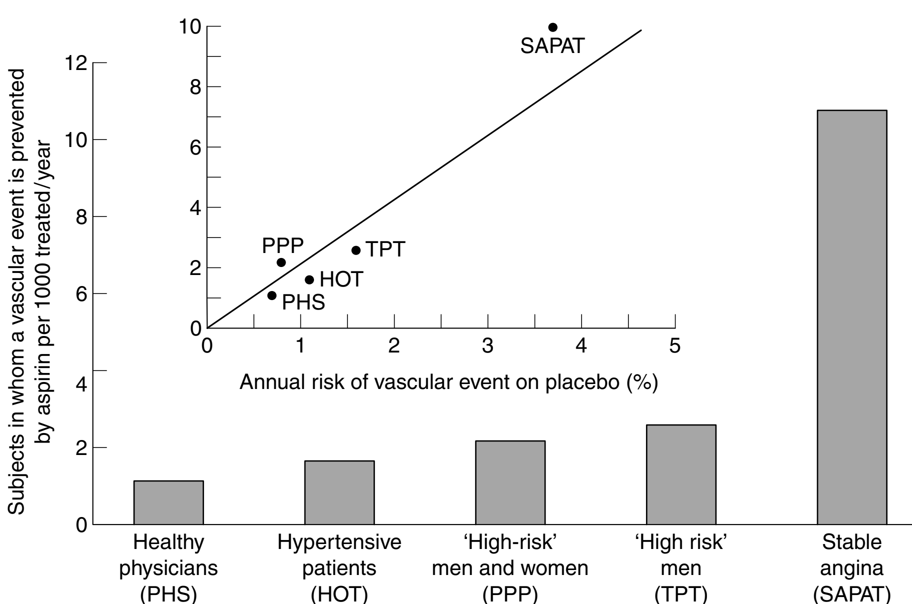


Fig. 61.2. Absolute benefit of aspirin in 'primary' prevention. Data were calculated from placebo-controlled aspirin trials in different settings characterized by variable cardiovascular risk, as noted on the abscissa. The absolute benefit of aspirin prophylaxis is reported on the ordinate as the number of subjects in whom an important vascular event (non-fatal MI, non-fatal stroke, or vascular death) is prevented by treating 1000 subjects with low-dose aspirin for 1 year.

i.e., approximately 1.5% per year. In this setting, an ischemic cardiac event would be avoided in two high-risk subjects by treating 1000 such men with aspirin for a year (Fig. 61.2). The recently reported Primary Prevention Project (PPP) has extended these findings to a group of 4495 men and women older than 50 years and with one or more cardiovascular risk factors recruited by 315 general practitioners in Italy[38]. After a mean follow-up of 3.6 years, the trial was prematurely stopped on ethical grounds and failed to demonstrate a statistically significant reduction in the primary endpoint (RR, 0.71; 95% CI, 0.48–1.04).

Quite similar results were obtained in the Hypertension Optimal Treatment study[39], in which 18 790 male and female patients with intensively treated hypertension were randomly allocated to aspirin, 75 mg daily, or placebo. Aspirin reduced major vascular events by 15% ($P = 0.03$) and all MI by 36% ($P = 0.002$), with no effects on stroke or cardiovascular mortality. Because of the low vascular risk of these well-treated hypertensive patients (only about 1.0% per year), a major cardiovascular event would be avoided in one to two patients by treating 1000 hypertensive men and women with low-dose aspirin therapy for a year (Fig. 61.2). If one compares these absolute benefits of aspirin prophylaxis with those achieved in the 'primary' prevention of MI in patients with stable chronic angina[28], it becomes apparent that the level of cardiovascular risk in the control population (i.e., those on placebo) represents a major determinant of the absolute benefit of antiplatelet therapy (Fig. 61.2).

These results do not support the widespread use of aspirin for 'primary' cardiovascular prophylaxis, because they clearly demonstrate that proper management of modifiable risk factors by current multifactorial strategies can reduce the actual risk of experiencing a major vascular event to a level where the additional benefit of aspirin does not clearly outweigh the risk of major bleeding complications (see below).

Adverse effects of aspirin

Aspirin does not cause a generalized bleeding abnormality unless it is given to patients with an underlying hemostatic defect, such as hemophilia, uremia, or that induced by anticoagulant therapy. Aspirin-induced impairment of primary hemostasis cannot be separated from its antithrombotic effect and is similarly independent of the dose[2,3].

The balance between preventing vascular occlusion and causing excess bleeding with aspirin depends critically on the absolute thrombotic vs. hemorrhagic risk of the patient. Thus, in individuals at very low risk for vascular occlusion (e.g., 1% per year), a very small absolute benefit is offset by exposure of a large number of healthy subjects

Cambridge Books Online © Cambridge University Press, 2009

Table 61.3. Benefit/risk ratio of antiplatelet prophylaxis with aspirin in different settings

Clinical setting	Benefit[a] (Number of patients in whom a major vascular event is avoided per 1000/year)	Risk[b] (Number of patients in whom a major GI bleeding event is caused per 1000/year)
Men at low to high cardiovascular risk	1–2	1–2
Essential hypertension	1–2	1–2
Chronic stable angina	10	1–2
Prior myocardial infarction	20	1–2
Unstable angina	50	1–2

Notes:
[a] Benefits are calculated from randomized trial data reviewed in this chapter and depicted in Figs. 61.1 and 61.2.
[b] Risks of upper GI bleeding are estimated from a background rate of 1 event per 1000 per year in the general population of non-users and a relative risk of 2.0 to 3.0 associated with aspirin prophylaxis[42]. Such an estimate assumes comparability of other risk factors for upper GI bleeding, such as age and concomitant use of NSAIDs, and may actually underestimate the absolute risk in an elderly population exposed to 'primary' prevention. The absolute excess of major bleeding complications in the 'primary' prevention trials reviewed in Table 61.2 ranged between 0.3 and 2.2 per 1000 patient–years.
Source: Reproduced with permission[3].

to undue bleeding complications. In contrast, in patients at high risk of cardiovascular or cerebrovascular complications (e.g., >5% per year), the substantial absolute benefit of aspirin prophylaxis clearly outweighs the risk (Table 61.3)[3]. For example, the absolute excess of major bleeds (i.e., those requiring transfusion) in acute MI is approximately 1/100th the absolute number of major vascular events avoided by aspirin therapy[4].

Hypertension has often been considered a contraindication to aspirin because of the concern that possible benefits in the prevention of cardiovascular events may be counterbalanced by an increased risk of cerebral bleeding. The results of the aspirin component of the Hypertension Optimal Treatment study are reassuring in this regard, since hypertensive patients whose BP was well controlled were protected from MI by aspirin without an increase in cerebral bleeds or strokes[39].

The overall risk of major extracranial and intracranial bleeds associated with antiplatelet drugs is difficult to assess in individual trials because their incidence is low, i.e., <1% per year, making detection of even a 50 to 60% relative increase in risk unrealistic in most trials of a few thousand patients.

Aspirin-induced GI toxicity, as detected in randomized clinical trials, appears to be dose related in the range of 30 to 1300 mg daily[41]. This is based largely on indirect comparisons of different trials and on a limited number of randomized, direct comparisons of different aspirin doses, as reviewed above. Such a dose–response relationship is thought to reflect at least two COX-1-dependent components, i.e., dose-dependent inhibition of COX-1 in the GI mucosa and dose-independent (within the range of examined doses) inhibition of COX-1 in platelets[3]. Thus, it is not surprising that the antithrombotic effect of aspirin can be dissociated, at least in part, from its most common side effect. However, even when administered at low doses, aspirin can cause serious GI bleeding, as reported in studies using 30 to 50 mg daily[31,32]. Because of the underlying prevalence of gastric mucosal erosions related to concurrent use of other NSAIDs and/or *Helicobacter pylori* infection in the general population, it should be expected that any antiplatelet dose of aspirin will cause more bleeding from pre-existing lesions than a placebo. Consistent with this mechanistic interpretation, the relative risk of hospitalization due to upper GI bleeding and/or perforation associated with low-dose aspirin therapy (mostly, 100 to 300 mg daily) is comparable to that of other antiplatelet agents and anticoagulants, i.e., 2.3 (95% confidence interval [CI], 1.7 to 3.2), 2.0 (95% CI, 1.4 to 2.7), and 2.2 (95% CI, 1.4 to 3.4), respectively, in a large population-based observational study[42]. A case-control study with hospital and community controls has examined the risks of hospitalization for bleeding peptic ulcer associated with three different regimens of aspirin prophylaxis. Odds ratios were raised for all doses of aspirin taken: 75 mg, 2.3 (95%CI, 1.2 to 4.4); 150 mg, 3.2 (1.7 to 6.5); 300 mg, 3.9 (2.5 to 6.3). It has been calculated that approximately 900 of the 10 000 episodes of ulcer bleeding occurring in people aged over 60 each year in England and Wales could be associated with, and ascribed to, prophylactic aspirin use[43]. A general change to lower doses (75 mg) of aspirin would not eliminate risks but, if these figures are soundly based, would reduce risk by about 40% compared with 300 mg and by 30% compared with 150 mg doses. Given that the mortality rate among patients who are hospitalized for NSAID-induced upper GI bleeding is about 5 to 10%[44], such a strategy could save a significant number of lives.

The widely held belief that enteric-coated and buffered varieties of aspirin are less likely to occasion major upper GI bleeding than plain tablets was tested in data from a multicentre case-control study[45]. The relative risks of

Cambridge Books Online © Cambridge University Press, 2009

upper GI bleeding for plain, enteric-coated, and buffered aspirin at average daily doses of ≤325 mg was 2.6, 2.7, and 3.1, respectively. At doses >325 mg, the relative risk was 5.8 for plain and 7.0 for buffered aspirin; there were insufficient data to evaluate enteric-coated aspirin at this dose level[45]. Thus, physicians who recommend aspirin in an enteric-coated or buffered form should not assume that these formulations are less likely to cause GI tract bleeding than plain aspirin.

Suppressing acid secretion is thought to reduce the risk of ulcers associated with regular use of NSAIDs. In patients who required continuous treatment with NSAIDs and who had ulcers or >10 erosions in either the stomach or duodenum, omeprazole healed and prevented ulcers more effectively than did ranitidine. In these patients, maintenance therapy with omeprazole was associated with a lower rate of relapse and was better tolerated than misoprostol. However, whether suppressing acid secretion might reduce GI toxicity associated with low-dose aspirin remains to be established.

Substantially less information is available concerning the risk of intracranial hemorrhage associated with aspirin use. In the Nurses' Health Study cohort of approximately 79 000 women aged 34 to 59 yr, infrequent use of aspirin (1 to 6 tablets per week) was associated with reduced risk of ischemic stroke, while high frequency of use (15 or more aspirin per week) was associated with increased risk of subarachnoid hemorrhage, particularly among older or hypertensive women[46]. In the overview of the Antithrombotic Trialists' Collaboration, the absolute excess of intracranial hemorrhage due to aspirin therapy is less than 1 per 1 000 patients per year in high-risk trials, with somewhat higher risks in patients with cerebrovascular disease[4].

Low-dose aspirin therapy has not been reported to affect renal function or blood pressure control, consistent with its lack of effect on renal prostaglandin synthesis. Moreover, aspirin 75 mg daily did not affect BP nor the need for antihypertensive therapy in intensively treated hypertensive patients[39]. The suggestion that the use of aspirin and other antiplatelet agents is associated with reduced benefit from enalapril in patients with left ventricular systolic dysfunction is not supported by the results of a large meta-analysis of MI trials[47].

Conclusions

Thus, in summary, inhibition of TXA_2-dependent platelet function by aspirin may lead to prevention of thrombosis as well as to excess bleeding. Assessing the net effect requires an estimation of the absolute thrombotic vs. hemorrhagic risk of the individual patient. In individuals at very low risk for vascular occlusion, a very small absolute benefit may be offset by exposure of very large numbers of healthy subjects to undue bleeding complications. As the risk of experiencing a major vascular event increases, so does the absolute benefit of antiplatelet prophylaxis with aspirin, as shown in Fig. 61.1, for a number of clinical settings in which the efficacy of the drug has been tested in randomized clinical trials[3]. Based on the results of >100 such trials, the antithrombotic effect of aspirin does not appear to be dose related, over a wide range of daily doses (30 to 1300 mg), an observation consistent with saturability of platelet COX inhibition at very low doses. In contrast, GI toxicity of the drug does appear to be dose related, consistent with dose- and dosing interval-dependent inhibition of COX activity in the nucleated lining cells of the GI mucosa. Thus, aspirin once daily is recommended in all clinical conditions in which antiplatelet prophylaxis has a favourable benefit/risk profile. Because of GI toxicity and its potential impact on compliance, physicians are encouraged to use the lowest dose of aspirin shown effective in each clinical setting[3].

References

1 Majerus PW. Arachidonate metabolism in vascular disorders. *J Clin Invest* 1983; 72: 1521–1525.

2 Patrono C. Aspirin as an antiplatelet drug. *N Engl J Med* 1994; 330: 1287–1294.

3 Patrono C, Coller B, Dalen JE et al. Platelet-active drugs: the relationships among dose, effectiveness, and side effects. *Chest* 2001; 119: 395–635.

4 Antithrombotic Trialists' (ATT) Collaboration. Prevention of death, myocardial infarction and stroke by antiplatelet therapy: collaborative meta-analysis of 266 trials involving 200,000 patients at high risk of occlusive vascular events. *BMJ* 2001 (in press).

5 Smith WL, Garavito RM, DeWitt DL. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J Biol Chem* 1996; 271: 33157–33160.

6 Loll PJ, Picot D, Garavito RM. The structural basis of aspirin activity inferred from the crystal structure of inactivated prostaglandin H_2 synthase. *Nature Struct Biol* 1995; 2: 637–643.

7 Cipollone F, Patrignani P, Greco A et al. Differential suppression of thromboxane biosynthesis by indobufen and aspirin in patients with unstable angina. *Circulation* 1997; 96: 1109–1116.

8 Patrignani P, Filabozzi P, Patrono C. Selective cumulative inhibition of platelet thromboxane production by low-dose aspirin in healthy subjects. *J Clin Invest* 1982; 69: 1366–1372.

Cambridge Books Online © Cambridge University Press, 2009

9 Pedersen AK, FitzGerald GA. Dose-related kinetics of aspirin: presystemic acetylation of platelet cyclo-oxygenase. *N Engl J Med* 1984; 311: 1206–1211.
10 Clarke RJ, Mayo G, Price P, FitzGerald GA. Suppression of thromboxane A_2 but not systemic prostacyclin by controlled-release aspirin. *N Engl J Med* 1991; 325: 1137–1141.
11 McAdam B, Keimowitz RM, Maher M et al. Transdermal modification of platelet function: an aspirin patch system results in marked suppression of platelet cyclooxygenase. *J Pharmacol Exp Ther* 1996; 277: 559–564.
12 Topper JN, Cai J, Falb D et al. Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively upregulated by steady laminar shear stress. *Proc Natl Acad Sci USA* 1996; 93: 10417–10422.
13 McAdam BF, Catella-Lawson F, Mardini IA et al. Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. *Proc Natl Acad Sci USA* 1999; 96: 272–277.
14 FitzGerald GA, Oates JA, Hawiger J et al. Endogenous biosynthesis of prostacyclin and thromboxane and platelet function during chronic administration of aspirin in man. *J Clin Invest* 1983; 71: 678–688.
15 Murata T, Ushikubi F, Matsuoka T et al. Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature* 1997; 388: 678–682.
16 Ridker PM, Cushman M, Stampfer MJ et al. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med* 1997; 336: 973–979.
17 FitzGerald GA, Patrono C. The Coxibs: Inhibitors of cyclooxygenase-2. *N Engl J Med* 2001; 345: 433-442.
18 Helgason CM, Hoff JA, Kondos GT et al. Platelet aggregation in patients with atrial fibrillation taking aspirin or warfarin. *Stroke* 1993; 24: 1458–1461.
19 Helgason CM, Tortorice KL, Winkler SR et al. Aspirin response and failure in cerebral infarction. *Stroke* 1993; 24: 345–350.
20 Helgason CM, Bolin KM, Hoff JA et al. Development of aspirin resistance in persons with previous ischemic stroke. *Stroke* 1994; 25: 2331–2336.
21 Weksler BB, Kent JL, Rudolph D et al. Effects of low dose aspirin on platelet function in patients with recent cerebral ischemia. *Stroke* 1985; 16: 5–9.
22 Buchanan MR, Brister SJ. Individual variation in the effects of ASA on platelet function: implications for the use of ASA clinically. *Can J Cardiol* 1995; 11: 221–227.
23 Berglund U, Wallentin L. Persistent inhibition of platelet function during long-term treatment with 75 mg acetylsalicylic acid daily in men with unstable coronary artery disease. *Eur Heart J* 1991; 12: 428–433.
24 Weber AA, Zimmermann KC, Meyer-Kirchrath J et al. Cyclooxygenase-2 in human platelets as a possible factor in aspirin resistance. *Lancet* 1999; 353: 900.
25 Vejar M, Fragasso G, Hackett D et al. Dissociation of platelet activation and spontaneous myocardial ischemia in unstable angina. *Thromb Haemost* 1990; 63: 163–168.
26 Maclouf J, Folco G, Patrono C. Eicosanoids and iso-eicosanoids: Constitutive, inducible and transcellular biosynthesis in vascular disease. *Thromb Haemost* 1998; 79: 691–705.
27 The RISC Group. Risk of myocardial infarction and death during treatment with low dose aspirin and intravenous heparin in men with unstable coronary artery disease. *Lancet* 1990; 336: 827–830.
28 Juul-Moller S, Edvardsson N, Jahnmatz B et al. Double-blind trial of aspirin in primary prevention of myocardial infarction in patients with stable chronic angina pectoris. *Lancet* 1992; 340: 1421–1425.
29 The SALT Collaborative Group. Swedish Aspirin Low-Dose Trial (SALT) of 75 mg aspirin as secondary prophylaxis after cerebrovascular ischaemic events. *Lancet* 1991; 338: 1345–1491.
30 Lindblad B, Persson NH, Takolander R et al. Does low-dose acetylsalicylic acid prevent stroke after carotid surgery? A double-blind, placebo-controlled randomized trial. *Stroke* 1993; 24: 1125–1128.
31 Diener HC, Cunha L, Forbes C et al. European Stroke Prevention Study: II. Dipyridamole and acetylsalicylic acid in the secondary prevention of stroke. *J Neurol Sci* 1996; 143: 1–13.
32 The Dutch TIA Trial Study Group. A comparison of two doses of aspirin (30 mg vs 283 mg a day) in patients after a transient ischemic attack or minor ischemic stroke. *N Engl J Med* 1991; 325: 1261–1266.
33 Farrel B, Godwin J, Richards S et al. The United Kingdom transient ischaemic attack (UK-TIA) aspirin trial: final results. *J Neurol Neurosurg Psychiatry* 1991; 54: 1044–1054.
34 Taylor DW, Barnett HJM, Haynes RB et al. Low-dose and high-dose acetylsalicylic acid for patients undergoing carotid endarterectomy: a randomised controlled trial. *Lancet* 1999; 353: 2179–2184.
35 Steering Committee of the Physicians' Health Study Research Group. Final report on the aspirin component of the ongoing Physicians' Health Study. *N Engl J Med* 1989; 321: 129–135.
36 Peto R, Gray R, Collins R et al. Randomised trial of prophylactic daily aspirin in British male doctors. *BMJ* 1988; 296: 313–316.
37 The Medical Research Council's General Practice Research Framework. Thrombosis prevention trial: randomised trial of low-intensity oral anticoagulation with warfarin and low-dose aspirin in the primary prevention of ischemic heart disease in men at increased risk. *Lancet* 1998; 351: 233–241.
38 Collaborative Group of the Primary Prevention Project (PPP). Low-dose aspirin and vitamin E in people at cardiovascular risk: a randomised trial in general practice. *Lancet* 2001; 357: 89–95.
39 Hansson L, Zanchetti A, Carruthers SG et al. Effects of intensive blood-pressure lowering and low-dose aspirin in patients with hypertension: principal results of the Hypertension Optimal Treatment (HOT) randomised trial. *Lancet* 1998; 351: 1755–1762.
40 Hennekens CH, Peto R, Hutchison GB et al. An overview of the British and American aspirin studies. *N Engl J Med* 1988; 318: 923–924.

Cambridge Books Online © Cambridge University Press, 2009

41 Roderick PJ, Wilkes HC, Meade TW. The gastrointestinal toxicity of aspirin: an overview of randomised controlled trials. *Br J Clin Pharmacol* 1993; 35: 219–226.

42 Garcia Rodriguez LA, Cattaruzzi C, Troncon MG et al. Risk of hospitalization for upper gastrointestinal tract bleeding associated with ketorolac, other nonsteroidal anti-inflammatory drugs, calcium antagonists, and other antihypertensive drugs. *Arch Intern Med* 1998; 158: 33–39.

43 Weil J, Colin-Jones D, Langman M et al. Prophylactic aspirin and risk of peptic ulcer bleeding. *BMJ* 1995; 310: 827–830.

44 Wolfe MM, Lichtenstein DR, Singh G. Gastrointestinal toxicity of nonsteroidal antiinflammatory drugs. *N Engl J Med* 1999; 340: 1888–1899.

45 Kelly JP, Kaufman DW, Jurgelon JM et al. Risk of aspirin-associated major upper-gastrointestinal bleeding with enteric-coated or buffered product. *Lancet* 1996; 348: 1413–1416.

46 Iso H, Hennekens CH, Stampfer MJ et al. Prospective study of aspirin use and risk of stroke in women. *Stroke* 1999; 30: 1764–1771.

47 Latini R, Tognoni G, Maggioni AP et al. Clinical effects of early angiotensin-converting enzyme inhibitor treatment for acute myocardial infarction are similar in the presence and absence of aspirin: systemic overview of individual data from nearly 96,712 randomized patients. *J Am Coll Cardiol* 2000; 35: 1801–1807.

Cambridge Books Online © Cambridge University Press, 2009

Pharmacology of ticlopidine and clopidogrel

Jean-Pierre Cazenave and Christian Gachet

Institut National de la Santé et de la Recherche Médicale U.311, Établissement Français du Sang-Alsace, Strasbourg, France

Introduction

The thienopyridines ticlopidine and clopidogrel have a closely related chemical structure and similar pharmacological activity[1–3]. In vivo, an active metabolite of these drugs is generated which selectively inhibits adenosine-5′diphosphate (ADP)-induced platelet aggregation and the ADP-mediated amplification of platelet responses to other agonists[4]. This selective effect is due to irreversible covalent modification by the active metabolite of the recently cloned purinergic P2 receptor of platelets, $P2Y_{12}$[5]. As a result, ticlopidine and clopidogrel were the first $P2Y_{12}$ inhibitors to be used clinically as antithrombotic drugs. These compounds have proved to be safe and highly efficacious in preventing the thrombotic complications of atherosclerotic vascular disease in the brain, heart and peripheral arteries. In addition, they are useful molecular probes of the $P2Y_{12}$ receptor and have permitted progress in our understanding of the role of ADP in platelet activation and vascular thrombosis[6].

ADP was identified in the early 1960s as a factor released from erythrocytes which influenced platelet adhesiveness to glass[7,8] and induced platelet aggregation[9,10]. The crucial role of ADP as a mediator of platelet activation was rapidly recognized in relation to both the physiological process of hemostasis and the development and extension of arterial thrombosis[11,12]. ADP is present at very high concentrations in platelet dense granules and is released when platelets are exposed to thrombin, collagen or thromboxane A_2 (TXA_2), thus reinforcing their aggregation[13–15]. Inhibitors of ADP-induced platelet aggregation have proved to be effective antithrombotic drugs in animal models and clinical trials[3,16,17]. ADP removing enzymes are also antithrombotic in experimental models[18,19], while patients with defects of ADP receptors or lacking ADP in their platelet dense granules experience bleeding[20]. Hence considerable evidence has accumulated to support the important role of platelets in atherosclerosis and its thrombotic complications (atherothrombosis) and has provided a rationale for the development of drugs which inhibit platelet functions.

The first thienopyridines were developed as antiphlogistic agents in 1972. Ticlopidine was found later in 1975 to be an inhibitor of platelet aggregation in man[21] and in 1980 was introduced to prevent platelet aggregation and thrombosis during extracorporeal circulation[22]. Since then several clinical trials have shown its beneficial effects in the secondary prevention of major vascular events in patients with a history of cerebrovascular disease and of major cardiac events after coronary artery stent insertion, as likewise in peripheral vascular disease. The further development of new thienopyridines led to the discovery in 1986 of the successor of ticlopidine, clopidogrel[23]. Clopidogrel was approved for clinical use in 1997 after a large phase III clinical trial (CAPRIE) involving 19 185 patients with a history of symptomatic atherosclerotic disease. The CAPRIE trial demonstrated an overall superiority of clopidogrel over aspirin in the prevention of vascular ischemic events, stroke, myocardial infarction and vascular death[24].

Activation of platelets by ADP (Gachet, C. and Cazenave, J.P. Chapter 9)

Addition of exogenous ADP to washed human platelets results in shape change, reversible aggregation in the presence of physiological concentrations of external ionized calcium and finally desensitization[6,25–27]. This primary aggregation is not associated with TXA_2 formation or secretion of granule contents[15,28]. Although itself a weak aggregating agent, ADP is a necessary cofactor for normal activation of platelets by other agonists such as thrombin or collagen which induce secretion of ADP from the dense

granules[13]. This might explain the well-known stabilization by ADP of platelet aggregates induced by thrombin[29,30]. In addition, low concentrations of ADP potentiate or amplify the effects of all agonists including weak agonists like serotonin[25], adrenaline[31] or chemokines[32,33].

Activation of platelets by ADP leads to extremely rapid Ca^{2+} entry and mobilization of intracellular Ca^{2+} stores and simultaneously to inhibition of adenylyl cyclase[6,26]. The molecular identity of the platelet receptors through which extracellular ADP elicits these physiological responses is known. The $P2Y_1$ metabotropic receptor, coupled to Gq protein/phospholipase C activation, is responsible for the mobilization of Ca^{2+} from internal stores and initiates aggregation[34–36], while the $P2Y_{12}$ metabotropic receptor is coupled to Gi_2 protein/adenylyl cyclase inhibition and is essential for full aggregation in response to ADP[5]. Coactivation of $P2Y_1$ and $P2Y_{12}$ is necessary for normal ADP-induced platelet aggregation[37–41], since inhibition of either receptor with selective antagonists[6] or by gene targeting is sufficient to block it[42–44].

Selective antagonists allow one to discriminate between the $P2Y_1$ and $P2Y_{12}$ receptors[6]. Adenosine-2′, 5′ bisphosphate (A2P5P), adenosine-3′, 5′ bisphosphate (A3P5P) and the structurally modified more potent compound MRS 2179 are selective antagonists of the $P2Y_1$ receptor[45–48]. The antiplatelet thienopyridine compounds ticlopidine, clopidogrel and its active metabolite[3,4], and CS-747[49] and its active metabolite R-99224[50] are specific inhibitors of the $P2Y_{12}$ receptor. The ATP analogues AR-C 66096 MX, AR-C67085 MX and AR-C 69931 MX are also selective $P2Y_{12}$ antagonists[17].

Pharmacology of thienopyridines

Ticlopidine and clopidogrel are structurally related thienopyridines which have similar pharmacological effects following oral or intravenous administration to animals or humans[2,3,51]. These drugs selectively inhibit the ex vivo platelet aggregation induced by ADP, collagen or low concentrations of thrombin[52,53]. The molecular target of ticlopidine and clopidogrel is the ADP receptor $P2Y_{12}$, as has been demonstrated experimentally by biochemistry, molecular biology and pharmacology[6].

Metabolism and pharmacokinetics

Ticlopidine's chemical name is 5-(2-chlorobenzyl)-4,5,6,7-tetrahydrothieno-[3,2-c] pyridine hydrochloride and its structure is shown in Fig. 62.1. It is administered orally to humans as a coated tablet containing 125 or 250 mg ticlopidine hydrochloride (Ticlid®). Ticlopidine is readily absorbed (90%) following a single oral dose of 250 mg and the peak plasma concentration is attained after 1 to 3 hours. Plasma levels of ticlopidine increase about threefold to reach a plateau when the drug is administered twice daily over a period of 2 to 3 weeks, due to drug accumulation. Most of the compound (>98%) is reversibly bound to plasma proteins, primarily albumin. Ticlopidine is a prodrug, which requires in vivo metabolism by the hepatic cytochrome P-450 enzymatic pathway to acquire pharmacological activity[1]. In rats, its metabolism is extensive and rapid, and, among the 13 metabolites identified, the 2-keto derivative found in bile is a more potent inhibitor, after further biotransformation, of ADP-induced platelet aggregation than ticlopidine itself[54]. The apparent elimination half-life of ticlopidine is 24 to 36 h after a single oral dose and up to 96 h after 14 days of repeated administration (250 mg twice daily). Thus the onset of the antiplatelet effect of ticlopidine is not immediate[2,55].

Clopidogrel hydrogen sulfate, methyl (+)-(S)-a-(2-chlorophenyl)-6,7-dihydrothieno [3,2-c] pyridin-5 (4 H)-acetate hydrogen sulfate, is a thienopyridine derivative chemically related to ticlopidine (Fig. 62.1). The presence of a carboxymethyl side group in the benzylic position gives the clopidogrel molecule increased pharmacological activity and a better safety profile than ticlopidine. Clopidogrel is a chiral drug with S-configuration[56]. The S-enantiomer is inactive in vitro[57] but is rapidly absorbed and undergoes extensive metabolism and metabolic activation in animals and humans. The R-enantiomer (SR 25989) (Fig. 62.1) does not inhibit ADP-induced platelet aggregation ex vivo and is devoid of antithrombotic activity[56]. The pharmacokinetics of clopidogrel are different from those of ticlopidine and its antiplatelet effect is more rapid, particularly when a loading dose is administered. An inhibition of ADP-induced platelet aggregation is detectable 2 h after oral administration of 400 mg clopidogrel and remains stable for up to 48 h. Repeated daily administration of 75 mg clopidogrel in human volunteers or atherothrombotic patients results in a steady-state maximal inhibition (40 to 60%), comparable to that afforded by 500 mg ticlopidine daily[23,55].

Clopidogrel hydrogen sulfate (Plavix®, Iscover®) is administered orally to humans as a coated tablet containing 75 mg clopidogrel base. After a single oral dose (up to 200 mg) or repeated doses (up to 100 mg), the unchanged molecule is not detected in peripheral blood. Clopidogrel is rapidly absorbed and undergoes extensive hepatic metabolization in experimental animals[58,59]. Its main systemic metabolite (85%), the carboxylic acid derivative SR 26334, is devoid of pharmacological activity and has a plasma elimination half-

Cambridge Books Online © Cambridge University Press, 2009

Ticlopidine

Clopidogrel
S-enantiomer

SR 25989
R-enantiomer

Clopidogrel active metabolite

Fig. 62.1. Molecular structures of the thienopyridines ticlopidine and clopidogrel. The S-enantiomer of clopidogrel is metabolized in the liver to an active metabolite, while its R-enantiomer (SR 25989) is metabolized to an inactive compound. PCR 4099 (not shown) is a racemic mixture of the two enantiomers of clopidogrel.

life of about 8 h. Clopidogrel and its major circulating inactive metabolite are reversibly bound (98% and 94% respectively) to plasma proteins. The metabolic pathway leading to formation of the active metabolite of clopidogrel and its mechanism of action have recently been elucidated using hepatocytes in culture. The prodrug clopidogrel is converted in the liver into 2-oxo-clopidogrel through a cytochrome P450–1A mono-oxygenase-dependent pathway. Hydrolysis of this 2-oxo-clopidogrel intermediate generates the active metabolite (Fig. 62.1), which has been identified as 2-{1-[(1S)-1(2-chlorophenyl)-2-methoxy-2-oxoethyl]-4-sulfanyl-3-piperidinyli-diene} acetic acid[4]. This highly labile active metabolite reacts irreversibly as a thiol reagent with the thiol of an amino acid, putatively one of the extracellular cysteines, of the $P2Y_{12}$ purinoceptor when platelets pass through the liver. Such a local, irreversible effect accounts for the irreversible tagging of the platelet $P2Y_{12}$ receptor, the duration of the specific inhibition for the life span of the platelet and the lack of antiaggregatory activity of the drug in plasma[4]. Since the corresponding metabolite of the R-enantiomer of clopidogrel (SR 25989) is inactive, this interaction with the $P2Y_{12}$ receptor is clearly highly dependent on its stereoisomery[56]. The active metabolite of clopidogrel is also highly labile in vitro. In contrast to the parent molecule, it acts on platelets and specifically inhibits all the effects induced by ADP through its binding to the

Cambridge Books Online © Cambridge University Press, 2009

$P2Y_{12}$ receptor. Finally, although ticlopidine and clopidogrel inhibit ADP-induced platelet aggregation by a similar mechanism, they must do so by generating two different active metabolites, as that of clopidogrel could not be derived from ticlopidine[4].

Mode of action on platelets

The pharmacological effects of ticlopidine and clopidogrel on platelet functions are identical and have been extensively studied in animals and humans[3]. It was reported from early work that administration of ticlopidine reduced platelet aggregation in response to many agonists[23]. This led to proposals that inhibition of the generation of prostanoids[60], activation of adenylyl cyclase and inhibition of phosphodiesterases[61] and/or inhibition of the fibrinogen receptor GPIIb–IIIa were the mechanisms responsible for the inhibition of platelet aggregation by thienopyridines. A so-called 'thrombasthenic state' of ticlopidine treated platelets was also suggested[62]. However, although ticlopidine inhibits the binding of fibrinogen to GPIIb–IIIa, this is not the consequence of a direct modification of the integrin[63]. It is now clear that ticlopidine and clopidogrel selectively block ADP-induced platelet aggregation and the ADP-mediated amplification of platelet responses to other agonists such as collagen or low concentrations of thrombin through specific and covalent modification of the $P2Y_{12}$ receptor[6,52,53,64,65]. The thienopyridine compounds have been used as molecular tools to probe the ADP-induced platelet activation/aggregation pathway and the elucidation of their mechanism of action on platelets is closely linked to the discovery of the ADP receptors and their signal transduction pathways.

Oral administration of the thienopyridines ticlopidine, clopidogrel or PCR 4099 (the racemic form of clopidogrel) to rats, rabbits or humans demonstrated that these compounds irreversibly inhibited the effects of ADP on platelets and prolonged the bleeding time. One important finding was that ticlopidine and clopidogrel selectively prevented the reduction by ADP but not by adrenaline of cAMP levels raised by stimulation of platelet adenylyl cyclase with PGE_1[64,65]. This suggested that the ADP receptor, at the time a pharmacological concept termed $P2_T$[66], could be coupled to a seven transmembrane domain G-protein. It was then shown that ADP stimulates the binding of GTPγS to G proteins and in particular to Gi_2, coupled to adenylyl cyclase, in human platelet membranes and that this effect was blocked by clopidogrel[67–69].

The thienopyridine compounds antagonize ADP-induced tyrosine phosphorylation of several platelet proteins[70] and dephosphorylation of the vasodilator-stimulated phosphoprotein VASP[39,71]. In contrast, they do not modify other specific receptor-mediated effects of ADP on platelets like shape change, intracellular calcium movements or the phosphorylation of proteins involved in these processes[6,35,72]. Ticlopidine and clopidogrel induce a dose-dependent reduction in the number of 2-methylthio-ADP binding sites on rodent or human platelets[73–76]. Binding studies using the poorly hydrolysable analogue [^{33}P]-2MeS–ADP have shown that human platelets have about 600 high-affinity binding sites for 2MeS–ADP (K_d ~5 nM)[76]. Clopidogrel treatment reduces the number of sites by 70% on rat platelets (from 1200 to 450) and leaves binding sites resistant to clopidogrel[74]. In these cells, whereas ADP-induced inhibition of adenylyl cyclase is completely blocked, this agonist can still elicit platelet shape change, a rise in intracellular calcium and at high concentrations transient residual aggregation[35]. Moreover, in patients with a congenital impairment of ADP-induced platelet aggregation but normal shape change, the platelets display very low levels of [^{33}P]-2MeS–ADP binding sites[76–78].

These pharmacological and clinical data fit the current model whereby ADP activates platelets through two G-protein coupled receptors, the $P2Y_1$ receptor which is responsible for initiation of aggregation and transient platelet aggregation and is insensitive to thienopyridine compounds, and the $P2Y_{12}$ receptor which is responsible for completion and amplification of the platelet responses to ADP and other agonists. The latter receptor is the molecular target of clopidogrel and has been shown to be defective in the patients cited above[5,6].

Antithrombotic activity in experimental thrombosis

The antithrombotic activity of ticlopidine and clopidogrel has been demonstrated in several animal species using models of arterial thrombosis where platelet aggregation plays a major part. As specific inhibitors of ADP-induced aggregation, the thienopyridines have thus contributed to recognition of the important role of ADP in arterial thrombosis. In various arterial models, clopidogrel exhibited a dose-dependent antithrombotic activity and was about 50 times more potent than ticlopidine. These compounds both strongly inhibited thrombosis induced by different methods in rats[12,79]. Experimental thrombosis on a collagen-coated arterioarterial shunt in rats was reduced by clopidogrel under conditions where aspirin was inefficient but hirudin more potent, thus demonstrating that thrombin generation contributed significantly to platelet activation[80]. Clopidogrel also inhibited thrombus formation when the shunt was coated with tissue factor and thrombin generation was important[81]. In rats, rabbits, dogs, pigs

Cambridge Books Online © Cambridge University Press, 2009

or non human primates, clopidogrel was able to reduce thrombosis and cyclic flow variation in stenosed and endothelium-injured arteries, to inhibit stent thrombosis induced by high shear stress and to prevent the thrombotic occlusion of vascular grafts or vessels with deep medial injury[79,82–87]. A number of these studies showed that clopidogrel was superior to aspirin, particularly as an adjuvant to prevent reocclusion after thrombolysis induced by streptokinase[88] or tissue-type plasminogen activator[89]. The antiaggregatory and antithrombotic properties of clopidogrel were, however, potentiated by aspirin in several experimental models of arterial injury (silk thread or stent in an arteriovenous shunt, electrical stimulation) and in models of myointimal proliferation leading to restenosis in normal and atherosclerotic rabbits[90].

Thrombin is the most potent platelet activating agent and its effects are not inhibited by antiplatelet drugs. Clopidogrel nevertheless diminished stasis-induced venous thrombosis in rats and rabbits, demonstrating that under low thrombogenic experimental conditions ADP-induced platelet activation plays a role in thrombin formation[91,92]. In similar low thrombogenic conditions, clopidogrel was able to reduce thrombin generation in platelet-rich plasma from rats[93], an effect comparable to the inhibition of tissue factor-induced coagulation of human blood by platelet GPIIb–IIIa inhibitors but not by aspirin[94].

Other pharmacological effects of thienopyridines

Antiplatelet effects of clopidogrel have been reported in vitro using high concentrations and long incubation times[95], but this action of the drug is not specific as it is not due to inhibition of the $P2Y_{12}$ receptor[57]. However, although the antithrombotic activity of thienopyridines has been essentially attributed to inhibition of ADP-induced platelet aggregation, other pharmacological effects have been described which may contribute to their antithrombotic properties. These include a direct inhibitory effect on megakaryocytes[96], a decrease in levels of circulating fibrinogen[97], a reduction of erythrocyte aggregability[98], stimulation of nitric oxide generation[99], relaxation of the thoracic aorta in rats[100] and inhibition of platelet-dependent tissue factor expression on endothelial cells[101].

Ticlopidine has been shown to inhibit the synthesis of fibronectin by human endothelial cells in culture[102], to slow the progression of non proliferative diabetic retinopathy[103,104] and to inhibit pulmonary metastases in mice[105,106]. It was recently demonstrated that SR 25989, the R-enantiomer of clopidogrel which lacks antiaggregatory and antithrombotic activity, inhibited endothelial cell proliferation and migration in a wound healing model, while ticlopidine was two times less active[107]. This effect of SR 25989 was attributed to an up-regulation of thrombospondin-1 expression[108]. The antiangiogenic properties of SR 25989 have been further demonstrated by its inhibition of spontaneous microvessel formation from rat aorta embedded in a fibrin gel and in vivo inhibition of pulmonary metastatic dissemination in mice[109].

Clinical pharmacology

Platelets contribute to the arterial thrombotic complications of atherosclerosis (atherothrombosis) through multiple mechanisms promoting platelet activation, thrombin generation, vasoconstriction, the procoagulant activity of endothelium and inflammation. These platelet effects increase when the arteries are stenosed and the blood flow is turbulent. It is now known that platelet activation involves different agonists and receptor pathways, alone or in combination, and hence it is important to select and use the appropriate antiplatelet drug to inhibit a specific pathway in this complex process of thrombosis.

Aspirin and other drugs acting on platelets have proved beneficial in the treatment and prevention of atherothrombosis[55]. In a meta-analysis of more than 50 secondary prevention trials in various groups of patients[110], aspirin was found to reduce vascular death by about 15% and non fatal vascular events by about 30%. It could be concluded from these results that aspirin, which specifically inhibits COX-1 and the thromboxane A_2 pathway, has important but limited antithrombotic properties and that antiplatelet drugs targeting other activation pathways such as ADP, thrombin or GPIIb–IIIa should be compared to it in clinical trials. A number of such trials have been reviewed recently[2,51,55,111–115] and have shown ticlopidine and clopidogrel to be at least equally as efficacious as aspirin in specific clinical conditions. Thus, clinical trials have confirmed the pathophysiological importance of ADP-induced platelet aggregation in thrombosis complicating human vascular disease involving the brain, heart and peripheral arteries.

Long term treatment with ticlopidine in patients with a history of a neurologic ischemic event has consistently demonstrated a significant benefit over placebo or aspirin[116,117]. Ticlopidine was also efficacious in the secondary prevention of adverse events in unstable angina[118], myocardial infarction[119], percutaneous coronary intervention[120] and intermittent claudication[119]. The drug did not however gain wide acceptance on account of its association with side effects like hypercholesterolemia, neutropenia

Cambridge Books Online © Cambridge University Press, 2009

and thrombocytopenia[121], aplastic anemia[122] or thrombotic thrombocytopenic purpura[123] and it was usually prescribed as an alternative to aspirin in most situations. Since the antiplatelet and antithrombotic effects of ticlopidine were potentiated by aspirin in animals[124] and human volunteers[125], ticlopidine was nevertheless used in combination with aspirin in high risk situations. A combination of ticlopidine and aspirin has been routinely employed to prevent thrombosis after coronary stent insertion and has proved to be superior to aspirin alone[126] or aspirin with warfarin anticoagulation[127]. As the risk of serious side effects still persists with use of ticlopidine, clinicians have tended to replace it by clopidogrel following publication of the results of a large phase III clinical trial (CAPRIE, clopidogrel vs. aspirin in patients at risk of ischemic events).

CAPRIE[24] enrolled 19 185 patients in three groups, all with an increased risk of recurrent ischemic events: individuals who had experienced a recent stroke or recent myocardial infarction or had symptomatic peripheral arterial disease. This was the first study to test the efficacity and safety of clopidogrel (75 mg/day) as compared to aspirin (325 mg/day) after a mean follow-up of 1.9 years. Clopidogrel was significantly more effective than aspirin. The annual event rates for aspirin and clopidogrel were 5.83% and 5.32%, respectively, giving a risk reduction of 8.7%. When the three groups of patients were analysed separately, the risk reduction was much greater (23.8%) in those with symptomatic peripheral arterial disease, suggesting that the benefit of clopidogrel may not be identical in different clinical situations. It might be that ADP is more important in platelet activation through contact with diseased peripheral arteries, whereas ADP and TXA_2 are equally involved in platelet activation when a plaque ruptures in a coronary artery.

The CAPRIE study provided important safety data for clopidogrel, which appeared to be equally as well tolerated as aspirin. There was notably no excess neutropenia in the clopidogrel group and although the frequencies of severe rash and diarrhea were higher in these patients, gastrointestinal discomfort and hemorrhage were more frequent in the aspirin group. Thrombotic thrombocytopenic purpura could occur within the first 2 weeks after initiation of clopidogrel, but to a lesser extent than with ticlopidine[128]. Thus, as clopidogrel is safer than ticlopidine and has a much more rapid onset of action, new strategies are being tested which include its association with other antiplatelet drugs to inhibit different pathways of platelet activation. Administration of a front-loaded dose of clopidogrel (300 mg) to atherosclerotic patients on aspirin achieved an antithrombotic effect within 2 hours during acute coronary syndromes and after coronary stenting[129,130]. Although combined treatment with clopidogrel and aspirin has significantly reduced early ischemic events following coronary stenting, thrombosis of the stent still occurs in up to 1% of patients. A recent study[131] showed that treatment with clopidogrel and aspirin at a higher continuous dose (150 mg/day) and loading dose (600 mg) was superior to the combination of standard clopidogrel (75 mg/day) and aspirin at a lower loading dose (300 mg)[129]. This regimen inhibited ADP-induced platelet aggregation and secretion but had no effect on the aggregation and degranulation induced by activation of the platelet thrombin receptor, PAR-1. The latter observation is highly relevant to the thrombotic complications occurring during acute coronary syndromes, after stent replacement or following fibrinolytic treatment, where thrombin generation is important and aspirin and thienopyridines are not efficacious. Other recent studies have explored the possibility of blocking platelet functions by combining ticlopidine with further antiplatelet drugs, including GPIIb–IIIa blockers such as abciximab, in order to improve the clinical outcome in patients undergoing coronary angioplasty[132] or experiencing acute myocardial infarction during fibrinolysis[133].

Conclusions

The thienopyridines ticlopidine and clopidogrel are the first antithrombotic drugs specific for the ADP purinoceptor $P2Y_{12}$ and are used in humans to prevent the thrombotic complications of vascular atherosclerosis. Clinical trials have demonstrated the safety of clopidogrel and its superiority over aspirin in preventing ischemic events, while association of clopidogrel with aspirin and other antiplatelet drugs inhibiting different platelet activation receptors can further reduce the incidence of thrombosis. The recent cloning of the $P2Y_{12}$ receptor offers the possibility of designing new $P2Y_{12}$ antagonists with improved specificity and efficacy. Finally, as ADP is a key mediator of platelet activation, the development of inhibitors targeting its second receptor on platelets, the $P2Y_1$ receptor, may provide other important antithrombotic agents for use alone or in combination with $P2Y_{12}$ antagonists.

Acknowledgements

The authors would like to thank all their coworkers and colleagues from the INSERM U.311 group for their contribution to the work exposed and J.N. Mulvihill for correcting the English of the manuscript.

Cambridge Books Online © Cambridge University Press, 2009

References

1 Defreyn G, Bernat A, Delebassee D, Maffrand JP. Pharmacology of ticlopidine: a review. *Semin Thromb Haemost* 1989; 15: 159–166.
2 Quinn MJ, FitzGerald DJ. Ticlopidine and clopidogrel. *Circulation* 1999; 100: 1667–1672.
3 Savi P, Herbert JM. Pharmacology of ticlopidine and clopidogrel. *Haematologica* 2000; 85: 73–77.
4 Savi P, Pereillo JM, Uzabiaga MF et al. Identification and biological activity of the active metabolite of clopidogrel. *Thromb Haemost* 2000; 84: 891–896.
5 Hollopeter G, Jantzen HM, Vincent D et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* 2001; 409: 202–207.
6 Gachet C. 2001; ADP receptors of platelets and their inhibition. *Thromb Haemost* 2001; 86.
7 Hellem A. The adhesiveness of human blood platelets in vitro. *Scand J Clin Lab Invest* 1960; 12: 1–117.
8 Gaarder A, Jonsen L, Laland S, Hellem A, PA O. Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. *Nature* 1961; 192: 531–532.
9 Ollgard E. Macroscopic studies of platelet aggregation: nature of an aggregating factor in red blood cells and platelets. *Thromb Diath Haemorrh* 1961; 6: 86–97.
10 Born GVR. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature* 1962; 194: 27–29.
11 Born GV. Adenosine diphosphate as a mediator of platelet aggregation in vivo: an editorial view. *Circulation* 1985; 72: 741–746.
12 Maffrand JP, Bernat A, Delebassee D, Defreyn G, Cazenave JP, Gordon JL. ADP plays a key role in thrombogenesis in rats. *Thromb Haemost* 1988; 59: 225–230.
13 Kinlough-Rathbone RL, Packham MA, Reimers HJ, Cazenave JP, Mustard JF. Mechanisms of platelet shape change, aggregation and release induced by collagen, thrombin or A 23,187. *J Lab Clin Med* 1977; 90: 707–709.
14 Reimers H. Adenine nucleotides in blood platelets. In Longenecker G: *The platelets: Physiology and Pharmacology*, Academic Press, Orlando, 1985; 85–98.
15 Kinlough-Rathbone RL, Mustard JF, Packham MA, Perry DW, Reiners HJ, Cazenave JP. Properties of washed human platelets. *Thromb Haemost* 1977; 37: 291–308.
16 Cusack NJ, Hourani SM. Platelet P2 receptors: from curiosity to clinical targets. *J Auton Nerv Syst* 2000; 81: 37–43.
17 Humphries RG. Pharmacology of AR-C69931MX and related compounds: from pharmacological tools to clinical trials. *Haematologica* 2000; 85: 66–72.
18 Zawilska KM, Born GV, Begent NA. Effect of ADP-utilizing enzymes on the arterial bleeding time in rats and rabbits. *Br J Haematol* 1982; 50: 317–325.
19 Marcus AJ, Broekman MJ, Drosopoulos JHF et al. Human ecto-ADPase/CD39: thromboregulation via a novel pathway. *Haematologica* 2000; 85: 53–57.
20 Cattaneo M, Gachet C. ADP receptors and clinical bleeding disorders. *Arterioscler Thromb Vasc Biol* 1999; 19: 2281–2285.
21 Thébault JJ, Blatrix CE, Blanchard JF, Panak EA. Effects of ticlopidine, a new platelet aggregation inhibitor in man. *Clin Pharmacol Ther* 1975; 18: 485–490.
22 Renner C, Guilmet D, Curtet JM. La ticlopidine en chirurgie cardiaque avec circulation extra-corporelle. *Nouv Presse Méd* 1980; 9: 3249–3251.
23 Herbert JM, Fréhel D, Vallée E. Clopidogrel, a novel antiplatelet and antithrombotic agent. *Cardiovasc Drug Rev* 1993; 11: 180–198.
24 CAPRIE Steering Committee. A randomised blinded trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE) *Lancet* 1996; 348: 1329–1339.
25 Macfarlane DE. Agonists and receptors: adenosine diphosphate. In Holmsen H: *Platelet Responses and Metabolism*, CRC Press, Boca Raton, Florida 1987; 19–36.
26 Mills DC. ADP receptors on platelets. *Thromb Haemost* 1996; 76: 835–856.
27 Baurand A, Eckly A, Bari N et al. Desensitization of the platelet aggregation response to ADP: differential down-regulation of the P2Y1 and P2cyc receptors. *Thromb Haemost* 2000; 84: 484–491.
28 Mustard JF, Perry DW, Kinlough-Rathbone RL, Packham MA. Factors responsible for ADP-induced release reaction of human platelets. *Am J Physiol* 1975; 228: 1757–1765.
29 Cattaneo M, Canciani MT, Lecchi A et al. Released adenosine diphosphate stabilizes thrombin-induced human platelet aggregates. *Blood* 1990; 75: 1081–1086.
30 Kinlough-Rathbone RL, Packham MA, Perry DW, Mustard JF, Cattaneo M. Lack of stability of aggregates after thrombin-induced reaggregation of thrombin-degranulated platelets. *Thromb Haemost* 1992; 67: 453–457.
31 Lanza F, Beretz A, Stierle A, Hanau D, Kubina M, Cazenave JP. Epinephrine potentiates human platelet activation but is not an aggregating agent. *Am J Physiol* 1988; 255: H1276–H1288.
32 Clemetson KJ, Clemetson JM, Proudfoot AE, Power CA, Baggiolini M, Wells TN. Functional expression of CCR1, CCR3, CCR4, and CXCR4 chemokine receptors on human platelets. *Blood* 2000; 96: 4046–4054.
33 Kowalska MA, Ratajczak MZ, Majka M et al. Stromal cell-derived factor-1 and macrophage-derived chemokine: 2 chemokines that activate platelets. *Blood* 2000; 96: 50–57.
34 Jin J, Daniel JL, Kunapuli SP. Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J Biol Chem* 1998; 273: 2030–2034.
35 Hechler B, Eckly A, Ohlmann P, Cazenave JP, Gachet C. The P2Y1 receptor, necessary but not sufficient to support full ADP- induced platelet aggregation, is not the target of the drug clopidogrel. *Br J Haematol* 1998; 103: 858–866.
36 Savi P, Beauverger P, Labouret C et al. Role of P2Y1 purinoceptor in ADP-induced platelet activation. *FEBS Lett* 1998; 422: 291–295.
37 Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB, Kunapuli SP. Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. *J Biol Chem* 1998; 273: 2024–2029.

Cambridge Books Online © Cambridge University Press, 2009

38 Daniel JL, Dangelmaier C, Jin J, Kim YB, Kunapuli SP. Role of intracellular signalling events in ADP-induced platelet aggregation. *Thromb Haemost* 1999; 82: 1322–1326.

39 Geiger J, Honig-Liedl P, Schanzenbacher P, Walter U. Ligand specificity and ticlopidine effects distinguish three human platelet ADP receptors. *Eur J Pharmacol* 1998; 351: 235–246.

40 Jantzen HM, Gousset L, Bhaskar V et al. Evidence for two distinct G-protein-coupled ADP receptors mediating platelet activation. *Thromb Haemost* 1999; 81: 111–117.

41 Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. *Proc Natl Acad Sci USA* 1998; 95: 8070–8074.

42 Fabre JE, Nguyen M, Latour A et al. Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y1-deficient mice. *Nat Med* 1999; 5: 1199–1202.

43 Léon C, Hechler B, Freund M et al. Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. *J Clin Invest* 1999; 104: 1731–1737.

44 Léon C, Freund M, Ravanat C, Bauran A, Cazenave JP, Gachet C. A key role of the P2Y1 receptor in tissue factor induced thrombin dependent acute thromboembolism. Studies in P2Y1 knockout mice and mice treated with a P2Y1 antagonist. *Circulation* 2001; 103: 718–723.

45 Boyer JL, Romero-Avila T, Schachter JB, Harden TK. Identification of competitive antagonists of the P2Y1 receptor. *Mol Pharmacol* 1996; 50: 1323–1329.

46 Boyer JL, Mohanram A, Camaioni E, Jacobson KA, Harden TK. Competitive and selective antagonism of P2Y1 receptors by N6-methyl 2′- deoxyadenosine 3′,5′-bisphosphate. *Br J Pharmacol* 1998; 124: 1–3.

47 Nandanan E, Jang SY, Moro S et al. Synthesis, biological activity, and molecular modeling of ribose- modified deoxyadenosine bisphosphate analogues as P2Y(1) receptor ligands. *J Med Chem* 2000; 43: 829–842.

48 Baurand A, Raboisson P, Freund M et al. Inhibition of platelet function by administration of MRS 2179, a $P2Y_1$ receptor antagonist. *Eur J Pharmacol* 2001; 412: 213–221.

49 Sugidachi A, Asai F, Ogawa T, Inoue T, Koike H. The in vivo pharmacological profile of CS-747, a novel antiplatelet agent with platelet ADP receptor antagonist properties. *Br J Pharmacol* 2000; 129: 1439–1446.

50 Sugidachi A, Asai F, Yoneda K et al. Antiplatelet action of R-99224, an active metabolite of a novel thienopyridine-type G(i)-linked P2T antagonist, CS-747. *Br J Pharmacol* 2001; 132: 47–54.

51 Sharis PJ, Cannon CP, Loscalzo J. The antiplatelet effects of ticlopidine and clopidogrel. *Ann Intern Med* 1998; 129: 394–405.

52 Féliste R, Delebassée D, Simon MF et al. Broad spectrum antiplatelet activity of ticlopidine and PCR 4099 involves the suppression of the effects of released ADP. *Thromb Res* 1987; 48: 403–415.

53 Cattaneo M, Akkawat B, Lecchi A et al. Ticlopidine selectively inhibits human platelet responses to adenosine diphosphate. *Thromb Haemost* 1991; 66: 694–699.

54 Picard-Fraire C. Pharmacokinetic and metabolic characteristics of ticlopidine in relation to its inhibitory properties on platelet function. *Agents Actions* 1984; suppl 15: 68–75.

55 Patrono C, Coller B, Dalen JE et al. Platelet-active drugs. The relationship among dose, effectiveness, and side effects. *Chest* 2001; 119: 39S–63S.

56 Reist M, Roy-De-Vos M, Montseny JP et al. Very slow chiral inversion of clopidogrel in rats: a pharmacokinetic and mechanistic investigation. *Drug Metabol Disposition* 2000; 28: 1405–1410.

57 Herbert JM, Savi P. Non-specific inhibition of ADP-induced platelet antiaggregation by clopidogrel in vitro. *Thromb Haemost* 1999; 82:156–157.

58 Savi P, Herbert JM, Pflieger AM et al. Importance of hepatic metabolism in the antiaggregating activity of the thienopyridine clopidogrel. *Biochem Pharmacol* 1992; 44: 527–532.

59 Savi P, Combalbert J, Gaich C et al. The antiaggregating activity of clopidogrel is due to a metabolic activation by the hepatic cytochrome P450–1A. *Thromb Haemost* 1994; 72: 313–317.

60 Ashida SI, Abiko Y. Mode of action of ticlopidine in inhibition of platelet aggregation in the rat. *Thromb Haemost* 1979; 41: 436–449.

61 Bonne C, Battais E. Ticlopidine and adenylate cyclase. *Agents Actions* 1984; suppl 15: 88–96.

62 Di Minno G, Cerbone AM, Mattiolo PL, Turco S, Lovine C, Mancini M. Functionally thrombasthenic state in normal platelets following the administration of ticlopidine. *J Clin Invest* 1985; 75: 328–338.

63 Gachet C, Stierle A, Cazenave JP et al. The thienopyridine PCR 4099 selectively inhibits ADP-induced platelet aggregation and fibrinogen binding without modifying the membrane glycoprotein IIb–IIIa complex in rat and in man. *Biochem Pharmacol* 1990; 40: 229–238.

64 Gachet C, Cazenave JP, Ohlmann P et al. The thienopyridine ticlopidine selectively prevents the inhibitory effects of ADP but not of adrenaline on cAMP levels raised by stimulation of the adenylate cyclase of human platelets by PGE1. *Biochem Pharmacol* 1990; 40: 2683–2687.

65 Defreyn G, Gachet C, Savi P, Driot F, Cazenave JP, Maffrand JP. Ticlopidine and clopidogrel (SR 25990C) selectively neutralize ADP inhibition of PGE1-activated platelet adenylate cyclase in rats and rabbits. *Thromb Haemost* 1991; 65: 186–190.

66 Gordon JL. Extracellular ATP: effects, sources and fate. *Biochem J* 1986; 233: 309–319.

67 Gachet C, Cazenave JP, Ohlmann P, Hilf G, Wieland T, Jakobs KH. ADP receptor-induced activation of guanine-nucleotide-binding proteins in human platelet membranes. *Eur J Biochem* 1992a; 207: 259–263.

68 Gachet C, Savi P, Ohlmann P, Maffrand JP, Jakobs KH, Cazenave JP. ADP receptor induced activation of guanine nucleotide binding proteins in rat platelet membranes – an effect selectively blocked by the thienopyridine clopidogrel. *Thromb Haemost* 1992b; 68: 79–83.

Cambridge Books Online © Cambridge University Press, 2009

69 Ohlmann P, Laugwitz KL, Nurnberg B et al. The human platelet ADP receptor activates Gi2 proteins. *Biochem J* 1995; 312: 775–779.
70 Savi P, Artcanuthurry V, Bornia J et al. Effect of clopidogrel treatment on ADP-induced phosphorylations in rat platelets. *Br J Haematol* 1997; 97: 185–191.
71 Geiger J, Brich J, Honig-Liedl P et al. Specific impairment of human platelet P2Y(AC) ADP receptor-mediated signalling by the antiplatelet drug clopidogrel. *Arterioscler Thromb Vasc Biol* 1999; 19: 2007–2011.
72 Hechler B, Léon C, Vial C et al. The P2Y1 receptor is necessary for adenosine 5′-diphosphate-induced platelet aggregation. *Blood* 1998; 92: 152–159.
73 Mills DC, Puri R, Hu CJ et al. Clopidogrel inhibits the binding of ADP analogues to the receptor mediating inhibition of platelet adenylate cyclase. *Arterioscler Thromb* 1992; 12: 430–436.
74 Savi P, Laplace MC, Herbert JM. Evidence for the existence of two different ADP-binding sites on rat platelets. *Thromb Res* 1994c; 76: 157–169.
75 Savi P, Laplace MC, Maffrand JP, Herbert JM. Binding of [^{3}H]-2-methylthio ADP to rat platelets – effect of clopidogrel and ticlopidine. *J Pharmacol Exp Ther* 1994; 269: 772–777.
76 Gachet C, Cattaneo M, Ohlmann P et al. Purinoceptors on blood platelets: further pharmacological and clinical evidence to suggest the presence of two ADP receptors. *Br J Haematol* 1995; 91: 434–444.
77 Nurden P, Savi P, Heilmann E et al. An inherited bleeding disorder linked to a defective interaction between ADP and its receptor in platelets. Its influence on glycoprotein IIb–IIIa complex function. *J Clin Invest* 1995; 95: 1612–1622.
78 Léon C, Vial C, Gachet C et al. The P2Y1 receptor is normal in a patient presenting a severe deficiency of ADP-induced platelet aggregation. *Thromb Haemost* 1999; 81: 775–781.
79 Bernat A, Mares AM, Defreyn G, Maffrand JP, Herbert JM. Effect of various antiplatelet agents on acute arterial thrombosis in the rat. *Thromb Haemost* 1993; 70: 812–816.
80 Freund M, Mantz F, Nicolini P et al. Experimental thrombosis on a collagen coated arterioarterial shunt in rats: a pharmacological model to study antithrombotic agents inhibiting thrombin formation and platelet deposition. *Thromb Haemost* 1993; 69: 515–521.
81 Léger P, Magues JP, Freund M, Cazenave JP, Boneu B. Comparison of the antithrombotic effects of heparin, hirudin and clopidogrel according to the nature of the thrombogenic surface in an arterioarterial shunt in rats. *Thromb Haemost* 1999; 82: 1203–1204.
82 Harker LA, Marzec UM, Kelly AB et al. Clopidogrel inhibition of stent, graft, and vascular thrombogenesis with antithrombotic enhancement by aspirin in nonhuman primates. *Circulation* 1998; 98: 2461–2469.
83 Makkar RR, Eigler NL, Kaul S et al. Effects of clopidogrel, aspirin and combined therapy in a porcine ex vivo model of high-shear induced stent thrombosis. *Eur Heart J* 1998; 19: 1538–1546.
84 Herbert JM, Dol F, Bernat A, Falotico R, Lale A, Savi P. The antiaggregating and antithrombotic activity of clopidogrel is potentiated by aspirin in several experimental models in the rabbit. *Thromb Haemost* 1998; 80: 512–518.
85 Samama CM, Bonnin P, Bonneau M et al. Comparative arterial antithrombotic activity of clopidogrel and acetyl salicylic acid in the pig. *Thromb Haemost* 1992; 68: 500–505.
86 Yao SK, Ober JC, McNatt J et al. ADP plays an important role in mediating platelet aggregation and cyclic flow variations in vivo in stenosed and endothelium-injured canine coronary arteries. *Circ Res* 1992; 70: 39–48.
87 Yao SK, McNatt J, Cui K et al. Combined ADP and thromboxane A2 antagonism prevents cyclic flow variations in stenosed and endothelium-injured arteries in nonhuman primates. *Circulation* 1993; 88: 2888–2893.
88 Herbert JM, Bernat A, Sainte-Marie M, Dol F, Rinaldi M. Potentiating effect of clopidogrel and SR 46349, a novel 5-HT2 antagonist, on streptokinase-induced thrombolysis in the rabbit. *Thromb Haemost* 1993; 69: 268–271.
89 Yao SK, Ober JC, Ferguson JJ et al. Clopidogrel is more effective than aspirin as adjuvant treatment to prevent reocclusion after thrombolysis. *Am J Physiol* 1994; 267: H488–H493.
90 Herbert JM, Tissinier A, Defreyn G, Maffrand JP. Inhibitory effect of clopidogrel on platelet adhesion and intimal proliferation after arterial injury in rabbits. *Arterioscler Thromb* 1993; 13: 1171–1179.
91 Bernat A, Vallee E, Maffrand JP, Gordon JL. The role of platelets and ADP in experimental thrombosis induced by venous stasis in the rat. *Thromb Res* 1988; 52: 65–70.
92 Herbert JM, Bernat A, Maffrand JP. Importance of platelets in experimental venous thrombosis in the rat. *Blood* 1992; 80: 2281–2286.
93 Hérault JP, Dol F, Gaich C, Bernat A, Herbert JM. Effect of clopidogrel on thrombin generation in platelet-rich plasma in the rat. *Thromb Haemost* 1999; 81: 957–960.
94 Butenas S, Cawthern KM, van't Veer C, Di Lorenzo ME, Leck JB, Mann KM. Antiplatelet agents in tissue factor-induced blood coagulation. *Blood* 2001; 97: 2314–2322.
95 Weber AA, Reimann S, Schror K. Specific inhibition of ADP-induced platelet aggregation by clopidogrel in vitro. *Br J Pharmacol* 1999; 126: 415–420.
96 Cazenave JP, Gachet C. Anti-platelet drugs: do they affect megakaryocytes? *Baillières Clin Haematol* 1997; 10: 163–180.
97 Mazoyer E, Ripoll L, Boisseau MR, Drouet L. How does ticlopidine treatment lower plasma fibrinogen? *Thromb Res* 1994; 75: 361–370.
98 Hayakawa M, Kuzuya F. Effects of ticlopidine on erythrocyte aggregation in thrombotic disorders. *Angiology* 1991; 42: 747–753.
99 De Lorgeril M, Bordet JC, Salen P et al. Ticlopidine increases nitric oxide generation in heart-transplant recipients: a possible novel property of ticlopidine. *J Cardiovasc Pharmacol* 1998; 32: 225–230.
100 Dol-Gleizes F, Mares AM, Savi P, Herbert JM. Relaxant effect of

Cambridge Books Online © Cambridge University Press, 2009

2-methyl-thio-adenosine diphosphate on rat thoracic aorta: effect of clopidogrel. *Eur J Pharmacol* 1999; 367: 247–253.

101 Savi P, Bernat A, Dumas A, Ait-Chekn L, Herbert JM. Effect of aspirin and clopidogrel on platelet-dependent tissue factor expression in endothelial cells. *Thromb Res* 1994; 73: 117–124.

102 Piovella F, Ricetti MM, Almasio P, Samaden A, Semino G, Ascari E. The effect of ticlopidine on human endothelial cells in culture. *Thromb Res* 1984; 33: 323–332.

103 The TIMAD Study Group. Ticlopidine treatment reduces the progression of proliferative diabetic retinopathy. *Arch Ophtalmol* 1990; 108: 1577–1583.

104 Guillausseau PJ. Preventive treatment of diabetic microangiopathy: blocking the pathogenic mechanisms. *Diabetes Metab* 1994; 20: 219–228.

105 Kohga S, Kinjo M, Tanaka K, Ogawa H, Ishimara M, Tanaka K. Effects of 5-(2-chlorobenzyl)-4,5,6,7-tetrahydrothieno[3,2-C]pyridine hydrochloride (Ticlopidine), a platelet aggregation inhibitor, on blood-borne metastases. *Cancer Res* 1981; 41: 4710–4714.

106 Bando H, Yamashita T, Tsubura E. Effects of antiplatelet agents on metastasis. *Grann* 1981; 75: 284–291.

107 Klein-Soyer C, Cazenave JP, Herbert JM, Maffrand JP. SR 25989 inhibits healing of a mechanical wound of confluent human saphenous vein endothelial cells which is modulated by standard heparin and growth factors. *J Cell Physiol* 1994; 160: 316–322.

108 Klein-Soyer C, Ceraline J, Orvain C, de la Salle C, Bergerat JP, Cazenave JP. Angiogenesis inhibitor SR 25989 upregulates thrombospondin-1 expression in human vascular endothelial cells and foreskin fibroblasts. *Biol Cell* 1997; 89: 295–307.

109 Mah-Becherel MCM, Céraline J, Deplanque G et al. Anti-angiogenic effects of the thienopyridine SR 25989 in vitro and in vivo in a murine pulmonary metastasis model. *Br J Cancer* 2001; In press.

110 Antiplatelet Trialists' Collaboration. Collaborative overview of randomized trials of antiplatelet therapy: I. Prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. *BMJ* 1994; 308: 81–106.

111 Steinhubl SR. Antiplatelet agents in cardiology: the choice of therapy. *Ann Thorac Surg* 2000; 70: S3–8.

112 Bhatt DL, Kapadia SR, Yadav JS, Topol EJ. Update on clinical trials of antiplatelet therapy for cerebrovascular diseases. *Cerebrovasc Dis* 2000; 10, suppl.5: 34–40.

113 Hankey GJ, Sudlow CLM, Dunbabin DW. Thienopyridines or aspirin to prevent stroke and other serious vascular events in patients at high risk of vascular disease? A systematic review of the evidence from randomized trials. *Stroke* 2000; 31: 1779–1784.

114 Jarvis B, Simpson K. Clopidogrel: a review of its use in the prevention of atherothrombosis. *Drugs* 2000; 60: 347–377.

115 Bennett JS. Novel platelet inhibitors. *Annu Rev Med* 2001; 52: 161–181.

116 Gent M, Blakely JA, Easton JD et al. The Canadian American Ticlopidine Study (CATS) in thromboembolic stroke. *Lancet* 1989; i: 1215–1220.

117 Hass WK, Easton JD, Adams HP et al. A randomized trial comparing ticlopidine hydrochloride with aspirin for the prevention of stroke in high-risk patients: Ticlopidine Aspirin Stroke Study Group. *N Engl J Med* 1989; 321: 501–507.

118 Balsano F, Rizzon P, Violi F et al. Antiplatelet treatment with ticlopidine in unstable angina: a controlled multicenter clinical trial: the Studio della Ticlopidina nell'Angina Instable Group. *Circulation* 1990; 82: 17–26.

119 Janzon L, Bergqvist D, Boberg J et al. Prevention of myocardial infarction and stroke in patients with intermittent claudication: effects of ticlopidine: results from STIMS, the Swedish Ticlopidine Multicentre Study. *J Intern Med* 1990; 227: 301–308.

120 White CW, Chaitman B, Knudson ML. Antiplatelet agents are effective in reducing the acute ischemic complications of angioplasty but do not prevent restenosis: results from the ticlopidine trial. *Coronary Artery Dis* 1991; 2: 757–767.

121 FitzGerald GA. Ticlopidine in unstable angina: a more expensive aspirin? *Circulation* 1990; 82: 296–298.

122 Yeh SP, Hsueh EJ, Wu H, Wangn YC. Ticlopidine-associated aplastic anemia. A case report and review of literature. *Ann Hematol* 1998; 76: 87–90.

123 Bennett CL, Davidson CJ, Raisch DW, Weinberg PD, Bennett RH, Feldman MD. Thrombotic thrombocytopenic purpura associated with ticlopidine in the setting of coronary artery stents and stroke prevention. *Arch Intern Med* 1999; 159: 2524–2528.

124 Herbert JM, Bernat A, Samama M, Maffrand JP. The antiaggregating and antithrombotic activity of ticlopidine is potentiated by aspirin in the rat. *Thromb Haemost* 1996; 76: 94–98.

125 Lecompte TP, Lecrubier C, Bouloux C et al. Antiplatelet effects of the addition of acetylsalicylic acid 40 mg daily to ticlopidine in human healthy volunteers. *Clin Appl Thromb Hemost* 1997; 3: 245–250.

126 Leon MB, Baim DS, Popma JJ et al. A clinical trial comparing three antithrombotic-drug regimens after coronary-artery stenting. Stent Anticoagulation Restenosis Study Investigators. *N Eng J Med* 1998; 339: 1665–1671.

127 More RS, Chauhan A. Antiplatelet rather than anticoagulant therapy with coronary stenting. *Lancet* 1997; 349: 146–147.

128 Bennett CL, Connors JM, Carwile JM et al. Thrombotic thrombocytopenic purpura associated with clopidogrel. *N Engl J Med* 2000; 342: 1773–1777.

129 Bertrand ME, Rupprecht HJ, Urban P, Gershlick AH. Double-blind study of the safety of clopidogrel with and without a loading dose in combination with aspirin compared with ticlopidine in combination with aspirin after coronary stenting: the clopidogrel aspirin stent international cooperative study (CLASSICS) *Circulation* 2000; 102: 624–629.

130 Helft G, Osende JI, Worthley SG et al. Acute antithrombotic effect of a front-loated regimen of clopidogrel in patients with atherosclerosis on aspirin. *Arterioscler Thromb Vasc Biol* 2000; 20: 2316–2321.

Cambridge Books Online © Cambridge University Press, 2009

131 Müller I, Seyfarth, M Rüdiger S et al. Effect of a high loading dose of clopidogrel on platelet function in patients undergoing coronary stent placement. *Heart* 2001; 85: 92–93.
132 Fredrickson BJ, Turner NA, Kleiman NS et al. Effects of abciximab, ticlopidine, and combined abciximab/ticlopidine therapy on platelet and leukocyte function in patients undergoing coronary angioplasty. *Circulation* 2000; 101: 1122–1129.
133 Peter K, Kohler B, Straub A et al. Flow cytometric monitoring of glycoprotein IIb/IIIa blockade and platelet function in patients with acute myocardial infarction receiving reteplase, abciximab, and ticlopidine. *Circulation* 2000; 102: 1490–1496.

Cambridge Books Online © Cambridge University Press, 2009

63

Platelet membrane glycoprotein (GP) IIb–IIIa antagonists and acute arterial thrombosis

David R. Phillips and Lisa Nannizzi-Alaimo

COR Therapeutics, Inc., San Francisco, CA, USA

Introduction

GP IIb–IIIa (αIIbβ3 in integrin nomenclature[1]), the most abundant protein on the platelet surface, is the primary receptor mediating platelet aggregation, a process central to acute arterial thrombosis and to hemostasis. Indeed, its central role in aggregation positions GP IIb–IIIa at the heart of thrombosis and has directed aggressive strategies into developing a new class of drugs, termed GP IIb–IIIa antagonists, which block the binding of adhesive proteins to GP IIb–IIIa thus preventing platelet aggregation. GP IIb–IIIa antagonists have been shown to efficiently regulate platelet function, and to have a therapeutic benefit in reducing the acute thrombotic complications associated with coronary artery disease[2–5]. The pivotal role that GP IIb–IIIa serves in platelet aggregation arises due to the dynamic nature of this receptor which displays several functional activities that are important not only in understanding how this receptor is involved in platelet aggregation and thrombosis, but also, ultimately, for understanding how GP IIb–IIIa antagonists can be more effectively utilized to optimize their antithrombotic activities.

This chapter summarizes the role of GP IIb–IIIa in platelet function and the use of GP IIb–IIIa antagonists for the regulation of platelet functions involved in acute arterial thrombosis. The themes to be developed are threefold. First, GP IIb–IIIa is involved in a wide spectrum of reactions affecting platelet function and vascular biology. For example, while the central role of GP IIb–IIIa in platelet aggregation and thrombosis is well known, its central role in platelet function also involves it in clot formation, fibrinolysis, vascular remodelling and inflammation. Many of these functions occur because GP IIb–IIIa is not a static receptor, but dynamic, mediating signal transduction processes within the platelet that can affect, for example, the procoagulant activity of platelet, the secretion of granule proteins and the release of inflammatory activities. Second, nuances involved in the activities of GP IIb–IIIa antagonists have been identified which has facilitated the therapeutic use of this class of drug. Third, because GP IIb–IIIa ligand binding and signalling affects several platelet functions, it can be anticipated that use of GP IIb–IIIa antagonists will have clinical benefits in addition to the prevention of occlusive thrombosis, for example in anticoagulation, the enhancement of fibrinolysis and the reduction of inflammation. Several reviews of GP IIb–IIIa antagonists have been published[6]: the emphasis here will be on concepts relevant to the affects of these antagonists on platelet physiology, concepts relevant to the clinical use of these antagonists and on recent advances.

GP IIb–IIIa in platelet aggregation

‘Inside-out’ GP IIb–IIIa signalling

GP IIb–IIIa is a member of the integrin family of adhesion receptors[1]. Integrins are widely distributed, found on almost all cells at least in some stage of development, and mediate a broad spectrum of cell–cell and cell–substratum interactions. Both subunits, GP IIb and GP IIIa, have large extracellular domains, single transmembrane domains, and relatively short cytoplasmic domains. The distribution of GP IIb–IIIa is restricted to platelets and cells of the megakaryocytic lineage. On unstimulated, discoid platelets as normally exist in circulation, GP IIb–IIIa is distributed between the plasma membrane (~80 000 copies per platelet), the alpha granule membrane (~40 000 copies per platelet) and the dense body membrane (trace amounts)[7,8]. Although GP IIb–IIIa on unstimulated platelets has low affinity for plasma proteins, platelet stimula-

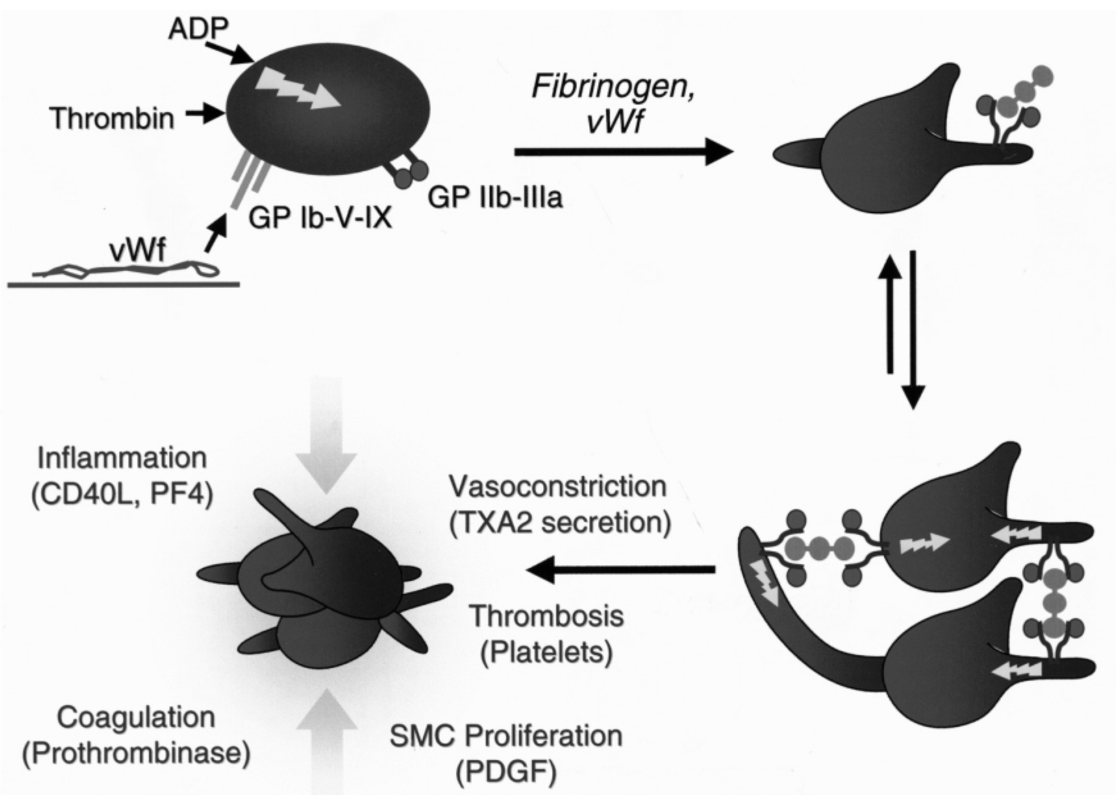


Fig. 63.1 (see also colour plate). Schematic illustrating the role of GP IIb–IIIa in platelet aggregation. On resting, discoid platelets the GP IIb–IIIa is present in an 'inactive' conformation and is unable to bind soluble fibrinogen. Upon activation by classical agonists such as collagen, thrombin or adenosine diphosphate (ADP) the platelet undergoes a shape change and the GP IIb–IIIa is activated such that it can now bind its soluble ligands, fibrinogen and von Willebrand factor (vWf). Platelet aggregation is mediated by the bivalent fibrinogen forming bridges between GP IIb–IIIa molecules on the surfaces of neighbouring, activated platelets.

tion, through a process known as 'inside-out' GP IIb–IIIa signalling, causes an upregulation of the affinity of GP IIb–IIIa for the soluble forms of several adhesive proteins, including fibrinogen, von Willebrand factor (vWf), vitronectin and fibronectin. Although the mechanism(s) responsible for inside-out GP IIb–IIIa signalling are not known, proteins that bind to the cytoplasmic domains of GP IIb and GP IIIa have been identified as have determinants of GP IIb and GP IIIa that maintain GP IIb–IIIa in the low affinity state[9]. Investigations into understanding how these factors regulate the affinity of GP IIb–IIIa for plasma proteins is being actively pursued. As shown in Fig. 63.1, see colour plate, platelet stimulation can be induced by a variety of stimuli, more commonly by thrombin generated in response to vascular injury, but ADP released from platelets or other cells in addition to vWf or collagen exposed in subendothelium, also play major roles in this process. Platelet stimulation, particularly when thrombin is the agonist, may also involve alpha granule secretion, causing up to a 50% increase in the exposure of GP IIb–IIIa. GP IIb–IIIa also shuttles between the plasma membrane and the alpha granule membrane, which is independent of platelet stimulation[10]. This shuttling allows for the packaging of fibrinogen in alpha granules during megakaryocytopoiesis[11] and for the uptake of GP IIb–IIIa ligands in mature platelets[12]. Nurden and coworkers[13] have shown the uptake of abciximab, a GP IIb–IIIa antagonist, which most likely occurred by this mechanism.

'Outside-in' GP IIb–IIIa signalling

The binding of fibrinogen and von Willebrand factor to GP IIb–IIIa on stimulated platelets is the primary driving force for the formation of initial platelet–platelet contacts to initiate aggregation. Both of these proteins are polyvalent, abundant, with high affinity for GP IIb–IIIa on stimulated platelets making them ideally suited to cross-link the

Cambridge Books Online © Cambridge University Press, 2009

membrane surfaces of interacting platelets. Recent data suggest that additional proteins are also involved in mediating platelet aggregation and thrombosis as these processes have been documented in a mouse strain lacking fibrinogen and vWf[14]. Platelet–platelet contacts during normal aggregation are initially reversible, but these contacts induce subsequent 'outside-in' GP IIb–IIIa signalling, which consolidates and stabilizes the platelet aggregate. Outside-in GP IIb–IIIa signalling can also be induced by the adhesion of unstimulated platelets to immobilized fibrinogen, a reaction that is mediated by fibrinogen binding to GP IIb–IIIa and facilitated by the structural change of fibrinogen that occurs because of its immobilization[15,16]. Outside-in GP IIb–IIIa signalling induces diverse functions that are critical to platelet physiology, since they are important not only to platelet aggregation, but also to responses of the vessel wall. Examples are numerous. Thromboxane A2 is generated which functions in an autocrine loop to stimulate platelets, but also has vasoconstrictive activity[17]. Metabolic events are induced, e.g. increased cytoplasmic Ca^{2+}, calpain activation, phosphorylation of platelet proteins and phospholipid metabolism, including the expression of phosphatidylinositol metabolites, which function during the platelet stimulatory process[9]. Alpha-granule proteins are secreted or expressed, many of which have been shown to have effects on the vasculature, e.g. P-selectin, PDGF, TGFβ and PF4. Dense body constituents are secreted, e.g. serotonin, ADP, which also appear to function in autocrine loops to induce further platelet stimulation and recruitment. New actin filaments form and cytoskeletal structures rearrange, events required for stable platelet adhesion, spreading and aggregation. A membrane 'PL scramblase' becomes activated resulting in the increased exposure of phosphatidyl serine and the increased expression of tenase and prothrombinase, enzyme complexes that are active in clot formation[18,19]. Membrane vesicles rich in these procoagulant activities bud off into microvesicles which are released from the platelet surface[20]. Translation of Bcl-3 and other proteins within the stimulated platelets is induced[21]. These secondary, outside-in platelet responses are important to consider in the present context as they predict, as discuss below, that GP IIb–IIIa antagonists will provide additional effects beyond simple inhibition of platelet aggregation and vessel occlusion. Recent work from our laboratory has established that GP IIIa becomes tyrosine phosphorylated during outside-in GP IIb–IIIa signalling, providing one mechanism by which GP IIb–IIIa transmits outside-in signals[22–24]. Inhibition of this pathway has been shown to prevent the formation of stable platelet aggregates[25].

Platelet agonist receptors

Platelet stimulation including inside out GP IIb–IIIa signalling occurs in response to a wide spectrum of physiological and pathophysiological conditions, e.g. vascular trauma to initiate normal hemostasis; atherosclerotic plaque rupture or percutaneous intervention to initiate thrombosis. Given the fundamental role of hemostasis for survival, it is not surprising that the pathways leading to GP IIb–IIIa activation are redundant. Redundancy is found on at least three levels. First, multiple agonists acting on distinct receptors are capable of initiating and augmenting the signal transduction pathways to induce the activation of the receptor function of GP IIb–IIIa and include soluble agonists, e.g. thrombin, ADP, epinephrine, serotonin and thromboxane A2, several adhesive proteins in the extracellular matrix, e.g. collagen, vWf and fibrinogen, high shear and a host of additional factors. This multiplicity of agonists broadens the physiological conditions capable of initiating platelet aggregation to cause thrombosis and hemostasis. It also means that therapeutic targeting of only one agonist, for example thrombin with heparin, or ADP with CD39, will only partially inhibit platelet stimulation, and may not have any inhibitory effect when high concentrations of other agonists are present. The second level of redundancy is that many of the primary agonists, e.g. thrombin, collagen and ADP, act on multiple receptors. In one example, ADP acts on at least three receptors on platelets: P2X1, a ligand-gated ion channel that allows for rapid Ca^{2+} influx; P2Y1, a Gq-coupled receptor that is linked to phospholipase C and mediates mobilization of Ca^{2+} from intracellular stores and platelet shape change, and the recently cloned P2Y12, a Gi-coupled receptor that inhibits adenylylcyclase and induces platelet aggregation[26]. In another example, collagen is known to bind to at least two receptors, the integrin $\alpha2\beta1$, and another membrane glycoprotein, GP VI[27,28]. A third example is thrombin, which stimulates platelets both through the PAR family of G-protein coupled receptors and through GP Ib–IX-V. The PAR receptors are cleaved by thrombin to initiate signal transduction reactions within platelets and GP IIb–IIIa activation and PAR-1 is recognized as the primary platelet receptor for thrombin in human platelets[29]. The GPIb–IX–V complex also appears to be involved in thrombin signalling, most likely because it contains a thrombin cleavage site (in GPV) and a high affinity thrombin binding site (in GP Ibα)[30,31]. The presence of multiple agonist receptors means that therapeutic targeting of only one of them, for example the P2Y12 with the thienopyridines, will only partially inhibit platelet stimulation. The third level of redundancy is the signal transduction pathway from agonist

Cambridge Books Online © Cambridge University Press, 2009

Table 63.1. Structures and uses for the three approved GP IIb–IIIa antagonists

Generic name	Eptifibatide	Tirofiban	Abciximab
Brand name	INTEGRILIN®	AGGRASTAT®	ReoPro®
Structure			Fab fragment of the chimeric human–murine monoclonal antibody 7E3
Affinity (K_D) for GP IIb–IIIa	120 nmol/l	15 nmol/l	5 nmol/l
Approved for use	PCI and unstable angina/ non Q-wave MI	unstable angina/non Q-wave MI	PCI

receptor stimulation through GP IIb–IIIa activation. Several pathways have been described, including pathway utilizing PKC and others involving phospholipase A2 (PLA2), which act in parallel to buttress inside-out signalling of GP IIb–IIIa to activate its receptor function. The presence of these multiple pathways means that therapeutic targeting of only one of them, for example, by targeting of cyclooxygenase with aspirin to inhibit the metabolism of arachidonate released by PLA2, or by targeting P2Y12 with one of the thienopyridines to block ADP-induced platelet stimulation, or by targeting thrombin with heparin or Factor Xa with low molecular weight heparin, will only partially inhibit platelet aggregation.

Clinical GP IIb–IIIa antagonists

Since GP IIb–IIIa is on the 'Final Common Pathway' leading to platelet aggregation, a term and concept first presented by Coller[32], antagonism of GP IIb–IIIa inhibits platelet aggregation irrespective of the agonist that stimulates platelets, irrespective of the agonist receptor, and irrespective of the stimulus–response coupling pathway used to activate the receptor function of GP IIb–IIIa. Table 63.1 lists the three approved GP IIb–IIIa antagonists. Abciximab (ReoPro®) is a Fab fragment of humanized 7E3, a GP IIb–IIIa monoclonal antibody[33]. Tirofiban (AGGRASTAT®) is a synthetic small molecule GP IIb–IIIa antagonist[34]. Eptifibatide (INTEGRILIN®) is a synthetic heptapeptide modelled on the active site of barbourin, a disintegrin found in the venom of the southeastern pigmy rattlesnake[35]. All are used by an i.v. mode of administration and bind directly to GP IIb–IIIa, both on stimulated or unstimulated platelets. While these agents do not affect agonist-induced platelet shape change, they function as antagonists of adhesive protein binding to GP IIb–IIIa, and therefore negate any upregulation of the receptor function of GP IIb–IIIa during platelet stimulation and block platelet aggregation induced by any of the platelet agonists. All are infused for short intervals (12–72 hours), but achieve a clinical benefit that persists months to years following therapy. Abciximab has been studied in the EPIC, EPILOG and CAPTURE trials and is approved for percutaneous revascularization; tirofiban was studied in the PRISM, PRISM Plus, and RESTORE trials and is approved for unstable angina/nonQ wave myocardial infarction; eptifibatide (INTEGRILIN®) was studied in the IMPACT II, PURSUIT and ESPRIT trials and is approved for both indications. The data supporting these approvals have been the subject of several reviews[5,36,37].

While the three parenterally available GP IIb–IIIa antagonists have many properties in common, important differences have been observed, many of which occur because these drugs have different affinities for GP IIb–IIIa (K_D = 5 nmol/l for abciximab; 15 nmol/l for tirofiban; and 120 nmol/l for eptifibatide[38]). The effect of affinity becomes important both during the infusion and in the recovery phase after infusion has been terminated. The three drugs are each infused at rates that yield plasma concentrations to achieve 70–90% occupancy of GP IIb–IIIa at steady state (less than 100% receptor occupancy is targeted to allow for sufficient hemostasis to prevent bleeding). During infusion, GP IIb–IIIa antagonists are in equilibrium between the plasma phase and GP IIb–IIIa on platelets. Accordingly, the highest affinity drug, abciximab, has the lowest unbound

Cambridge Books Online © Cambridge University Press, 2009

pool during infusion. The low unbound pool abciximab becomes important in individuals with high platelet count and may account for the variable inhibition of platelet aggregation observed with this drug[39,40]. The lower affinities of eptifibatide and tirofiban suggest that they have a faster off-rate from GP IIb–IIIa, and indeed, platelet function recovers faster when infusion of these drugs is discontinued compared to that which occurs with abciximab (2–4 hours for tirofiban and eptifibatide vs. 12–24 hours for abciximab). The unbound pools of all drugs are rapidly cleared. Even with abciximab, in the absence of the unbound pool, normal platelet aggregation can be observed, even with the persistent occupancy of the majority of the platelet surface GP IIb–IIIa 48 hours following the infusion of this drug[41]. Since it has not been shown that any GP IIb–IIIa antagonist provides any clinical benefit in the absence of an unbound pool, i.e. after infusion has been discontinued, it is not known whether the difference in rates of drug clearance have any effect on therapeutic efficacy or safety.

Effect of GP IIb–IIIa antagonists on platelet aggregation

The relative effects of GP IIb–IIIa antagonists on platelet aggregation has been the subject of extensive experimentation and of correspondingly much debate. The question is usually to determine the relative activities of the various antagonists in blocking platelet aggregation. All antagonists, added in sufficient quantity, are capable of totally blocking platelet function. The question, therefore, can be restated to ask 'how much is platelet aggregation inhibited when these drugs are administered to patients?' Studies that attempt to address this question by adding GP IIb–IIIa antagonists in vitro[42] are difficult to interpret as it is almost impossible to arrive at the concentration of drug that is achieved, in patients, during the bolus and steady-state phases of drug therapy. Because all antagonists are in equilibrium during the infusion phase of drug administration, dilution of samples prior to analysis characteristically cannot be done as this shifts the equilibrium and alters apparent inhibitory activities.

Three factors affect the apparent aggregation inhibitory activity of GP IIb–IIIa antagonists: the anticoagulant used for blood collection; the agonist used for platelet stimulation; and, the device used to measure platelet aggregation.

Anticoagulant effect on platelet aggregation

GP IIb–IIIa contains five divalent cation binding sites and the affinities of these sites for Ca^{2+} predict that all are occupied by this divalent cation when platelets are suspended in plasma[43]. Divalent cation binding is reversible and chelation of extracellular divalent cations removes Ca^{2+} from GP IIb–IIIa, dramatically affecting its structure, its binding of adhesive proteins and its binding of antagonists. For example, suspension of platelets in solutions containing ionized calcium concentrations of 40 to 50 μmol/l, as achieved in citrate-anticoagulated blood, causes partial removal of Ca^{2+} from GP IIb–IIIa. These effects of citrate not only cause a loss of fibrinogen-binding activity[44] but also increases the activity of GP IIb–IIIa antagonists in blocking platelet aggregation[45]. Because of these effects, platelet assays relying on GP IIb–IIIa function may not be valid when performed in buffers containing less than the 1.1 mmol/l Ca^{2+} normally found in plasma. One illustration of this effect was seen in the pharmacodynamic analysis of eptifibatide. The reduced Ca^{2+} caused by citrate anticoagulation not only increased the binding of eptifibatide, but also decreased the binding of fibrinogen. Since citrate anticoagulation was used for the ex vivo analysis of platelet aggregation by eptifibatide in the IMPACT II trial[46], the apparent inhibitory activity of eptifibatide was markedly overestimated. This analysis led to a dose that gave 40–50% inhibition of platelet aggregation at steady state, vs. the 80% inhibition that had been targeted in the IMPACT II trial[45]. When PPACK-anticoagulated blood was used to analyse the inhibitory activity of eptifibatide as was done in the subsequent PURSUIT[47] and ESPRIT[48] trials, markedly improved therapeutic efficacy was observed. Recent studies have shown that the apparent inhibitory activities of abciximab, tirofiban and other GP IIb–IIIa antagonists are also overestimated, although less so, in blood anticoagulated with citrate[49]. Thus, when inhibitory activities are compared between trials, or even within the same trial, the data can only be compared in blood anticoagulated by a mechanism which maintains the Ca^{2+} ion concentration that exists in blood. PPACK is preferable to heparin for this purpose, as heparin can induce platelet stimulation in some samples. Maintenance of plasma levels of Ca^{2+} during analysis also provides the most accurate estimate of in vivo platelet aggregation inhibitory activity.

Agonist effect on platelet aggregation

The 'strength' of the platelet agonist affects the amount of receptor competent GP IIb–IIIa recruited to the platelet surface and therefore the apparent inhibitory activity of any GP IIb–IIIa antagonist. Potent agonists like thrombin cause most of the alpha granule GP IIb–IIIa to be recruited with prebound fibrinogen to the platelet surface, while

Cambridge Books Online © Cambridge University Press, 2009

weak agonists like ADP only marginally increase the platelet surface GP IIb–IIIa[50,51]. This alpha granule pool of GP IIb–IIIa is functionally important as thrombin-stimulation of platelets in which the plasma membrane GP IIb–IIIa has been rendered inactive[52] or have been blocked with abciximab in the absence of the unbound pool[41] can still induce sufficient functional GP IIb–IIIa to the platelet surface to support platelet aggregation[53,54]. Alpha-granule secretion also induces the expression of P-selectin, a protein with a proven role in platelet function, to the platelet surface[55]. Pharmacodynamic studies consistently show that increased amounts of all three GP IIb–IIIa antagonists are required to block platelet aggregation by thrombin, the more potent platelet agonist, than by ADP[56]. For example, a lower amount of eptifibatide is required to inhibit aggregation induced by ADP (IC50 = 570 nM in PPACK anticoagulated PRP) than by thrombin (IC50 = 1190 nM), illustrating that increased amounts of GP IIb–IIIa antagonism is required to block platelet aggregation by the more potent platelet agonist[57]. Yet other studies show that the amount of antagonist required to inhibit platelet aggregation is proportional to the concentration of agonist used for platelet stimulation[49]. Since both thrombin and ADP (as well as collagen) are known to induce thrombosis in vivo, all are relevant to therapy. It is not known, however, which is more relevant, or is it known whether the importance of any agonist differs with different thrombotic conditions. These considerations illustrate that GP IIb–IIIa antagonists can only be compared when they are also measured using the same concentrations of the same agonists.

The recovery of platelet function following cessation of antagonist infusion is also differentially affected by agonists. Clinical studies have shown that thrombin-induced, but not ADP-induced platelet aggregation, is rapidly restored following discontinuation of infusion of high affinity GP IIb–IIIa antagonists like abciximab, or the synthetic GP IIb–IIIa antagonist L-738,167[55,58]. The unbound pools of these antagonists, but not the platelet bound pools, are rapidly cleared. Since thrombin, but not ADP, induces maximal surface expression of the alpha granule pool of GP IIb–IIIa, thrombin-induced aggregation is more rapidly restored.

Monitoring the effects of GP IIb–IIIa antagonists

Platelet aggregation has traditionally been measured by the rate of increased light transmittance through a stirred suspension of platelets (LTA). This method has been the most widely used for monitoring the aggregation inhibitory activities of GP IIb–IIIa antagonists and has been correlated to antithrombotic activities in animal models and in clinical studies. Using LTA, the targeted receptor occupancies of 70–90% nearly completely inhibit platelet aggregation induced by ADP but have less effect on platelet aggregation induced by TRAP, a synthetic peptide that activates platelets through the thrombin receptor. Because of this consideration, TRAP is preferred over ADP as the platelet agonist when the upper end of the dose–response curve for GP IIb–IIIa antagonists is being examined.

Because LTA is cumbersome and may not measure platelet aggregation under conditions that have in vivo relevancy such as high shear, attempts have been made to develop additional devices for this purpose[59]. One is the Cone and Platelet Analyser (CPA) which relies on flow-dependent platelet deposition onto a collagen-coated orifice. Another is the rapid platelet function Analyser (RPFA), which characteristically measures the ability of TRAP-activated platelets to aggregate with fibrinogen-coated beads in citrate anticoagulated PRP. The differences in anticoagulant, agonist and shear undoubtedly contribute to the differences observed in the analyses obtained with these devices[60]. Although the CPA and RPFA devices have the advantage of simplicity of use and point of care application, these methods have not yet been validated with respect to antithrombotic activities and clinical outcomes. Accordingly, it is not yet clear how the differences observed with these three methodologies will be used to optimize the extent of platelet aggregation inhibition required to optimize antithrombotic activity and minimize bleeding using GP IIb–IIIa antagonists.

Integrin cross-reactivity of GP IIb–IIIa antagonists

Tirofiban and eptifibatide were designed to specifically inhibit GP IIb–IIIa. In contrast, abciximab has been reported to also bind to and antagonize the receptor functions of two other integrins, $\alpha_v\beta3$ and Mac-1 (αMβ2, CD11b/CD18). It has been speculated that these integrin cross-reactivities of abciximab might provide a clinical advantage as $\alpha_v\beta3$ has been implicated in smooth muscle cell proliferation and vascular remodelling following percutaneous intervention and Mac-1 has been shown to be involved in the acute responses of leukocytes within the vasculature[61]. However, rigorous analysis of the binding properties of abciximab for these other integrins and close inspection of the clinical data suggests otherwise. αvβ3 is upregulated on smooth muscle cells and may modulate smooth muscle cell and endothelial cell replication[62].

Cambridge Books Online © Cambridge University Press, 2009

Because experimental vascular injury-induced intimal hyperplasia models show that inhibition of $\alpha v\beta 3$ reduces smooth muscle cell proliferation[63,64], abciximab was analysed for its ability to reduce the incidence of restenosis. While EPIC, the initial Phase III trial of abciximab showed that there was a reduction in the need for revascularization procedures due to the administration of abciximab, subsequent clinical trials[65] and studies of nonhuman primates[66] specifically designed to examine the antirestenotic activity of abciximab failed to demonstrate that this drug affected late loss following percutaneous intervention. Recent studies demonstrated antagonism of yet another integrin, $\alpha 5\beta 1$, in addition to $\alpha v\beta 3$ is required to block migration of smooth muscle cells on fibrin matrices[67], suggesting a reason for these results. Yet another reason is that the short interval of abciximab administration, 12 hours, and the rapid clearance of the unbound pool after infusion is terminated, does not coincide with the up-regulation of $\alpha v\beta 3$. Because experimental models showed that gene targeting of Mac-1 inhibited restenosis in mice[68] and that Mac-1 is required for leukocyte adhesive reactions[69], it has also been suggested that the Mac-1 binding property of abciximab may provide an anti-inflammatory benefit[61]. However, in order for abciximab to bind Mac-1, this integrin must first be activated[70]. Most leukocytes circulate with Mac-1 in an inactive state: no abciximab can be detected on circulating leukocytes during abciximab therapy[71]. Since abciximab binds to GP IIb–IIIa on unstimulated platelets with 40-fold higher affinity than to stimulated Mac-1, and since fibrinogen, present at approximately 1000 fold greater concentration than the unbound pool of abciximab, also binds to Mac-1 once it is stimulated, it is unlikely that effective antagonism of Mac-1 can be achieved under the clinical administration of this drug. Thus, while a clinical benefit may result by targeting of integrins such as $\alpha v\beta 3$ and Mac-1 in acute vascular conditions, possibly by the development of specific antagonists that can be used at concentrations that saturate receptors for prolonged periods of time, the existing data do not support the notion that any of the three approved parenteral GP IIb–IIIa antagonists provide a clinical benefit by any mechanism other than those resulting from the antagonism of GP IIb–IIIa.

GP IIb–IIIa antagonists and LIBS epitope expression

All GP IIb–IIIa ligands (adhesive proteins such as fibrinogen, GP IIb–IIIa antagonists such as tirofiban and eptifibatide, including selected antibodies such as abciximab) induce a conformational change in GP IIb–IIIa that can be detected by the expression of LIBS (ligand-induced binding site) antibodies. The repertoire of LIBS epitopes expressed by these ligands, however, differs, and LIBS antibodies have been identified that identify these changes in GP IIb–IIIa. For example, while eptifibatide and tirofiban but not lamifiban[72,73] induce the binding of the LIBS antibody termed D3, all these GP IIb–IIIa antagonists induce the binding of mAb15–758[74]. Abciximab binding to GP IIb–IIIa induces the expression of additional LIBS epitopes[75]. Presumably, additional changes in GP IIb–IIIa occur that have not yet been detected by any of the known LIBS antibodies.

LIBS epitope expression is increasingly recognized for its potential impact in limiting the use of GP IIb–IIIa antagonists particularly in long-term, oral administration. Reports have appeared indicating thrombocytopenia in a few individuals as a complication of exposure to GP IIb–IIIa antagonists[76–78]. Hypotheses for this phenomenon are that the LIBS epitopes expressed either cause platelet clearance directly or induce the production of antibodies to the LIBS epitopes which secondarily cause platelet clearance by an immune mechanism. Evidence for this comes from animal studies where thrombocytopenia was observed in both chimpanzee and Rhesus monkeys and this condition corresponded to the presence of drug-dependent antibodies against GP IIb–IIIa[79]. Accumulation of information on this point for different GP IIb–IIIa antagonists could identify LIBS epitopes critical for this response. Thrombocytopenia induced by the three parenteral GP IIb–IIIa antagonists is infrequent, and not routinely associated with severe bleeding complications[80,81].

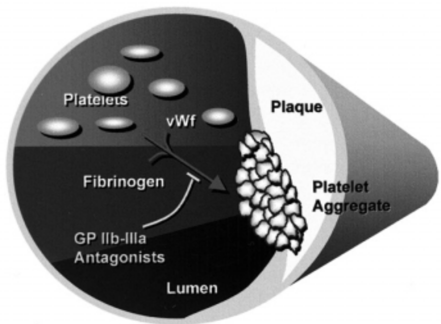
GP IIb–IIIa in coagulation, inflammation and vascular cell proliferation

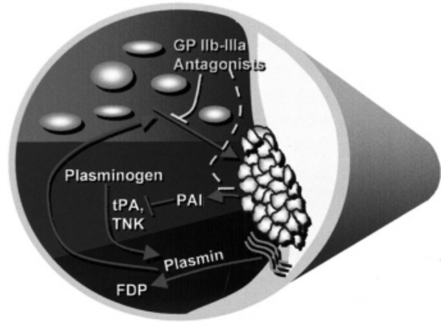
Recent data suggest that GP IIb–IIIa antagonists may have activities in addition to inhibition of platelet aggregation. The data summarized below, and in Fig. 63.2, see colour plate, suggest that these additional activities include anticoagulant, fibrinolytic, and anti-inflammation.

Coagulation

It is well known that the presence of platelets in blood shortens the time required for clot formation as platelets provide a surface for the assembly of the tenase complex which activates Factor X to Factor Xa, and the prothrombinase complex which converts prothrombin to thrombin.

Cambridge Books Online © Cambridge University Press, 2009





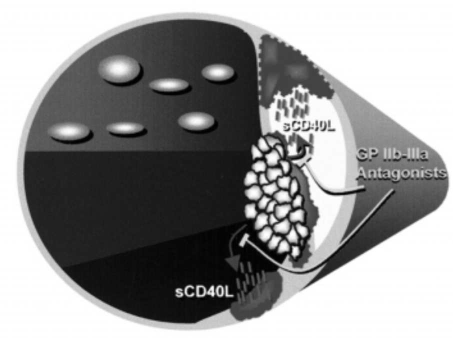


Fig. 63.2 (see also colour plate). Diagrams depict the multiple benefits of GP IIb–IIIa antagonists. (*a*) GP IIb–IIIa antagonists block platelet aggregation and recruitment to prevent thrombosis. (*b*) The prothrombinase complex (FVa, FXa) is predominantly formed on the surface of aggregated platelets. The anticoagulant activity of GP IIb–IIIa antagonists can be attributed to their antiaggregatory activity. (*c*) GP IIb–IIIa antagonists inhibit platelet aggregation induced by plasmin and thrombin which are generated during thrombolysis. These antagonists also inhibit the aggregation-induced release of PAI-1, an inhibitor of fibrinolysis. The apparent 'fibrinolytic' activities of GP IIb–IIIa antagonists can be attributed to these synergistic activities with fibrinolytic agents. (*d*) Platelet aggregation induces the expression and release of the potent proinflammatory protein CD40L. Inhibition of platelet adhesion to vascular cells and sCD40L release from platelets by GP IIb–IIIa antagonists may allow this class of drugs to be anti-inflammatory.

It has been shown that GP IIb–IIIa antagonists reduced tissue factor induced thrombin formation indicating that clot formation and GP IIb–IIIa may be linked[82–85]. These findings raise the interesting possibility that GP IIb–IIIa antagonists may decrease thrombin formation at sites of vascular injury. In support of this suggestion, ex vivo samples have prolonged clot formation time when obtained from patients receiving GP IIb–IIIa antagonists[86]. Although unstimulated platelets have poor clot promoting activity, activated, adherent platelets assemble prothrombinase and Factor Xase on their surface to greatly enhance the rate of thrombin generation[87]. As shown in Fig. 63.2(*b*), see colour plate, GP IIb–IIIa antagonists actually inhibit thrombin generation[84]. Since aggregation induces platelet activation through outside-in GP IIb–IIIa signalling, this may account for the ability of the three GP IIb–IIIa antagonists to reduce thrombin formation[88]. An alternative hypothesis is derived from the experiments of Byzova and Plow[89], who showed that prothrombin binds to GP IIb–IIIa on unstimulated platelets. These authors speculate that prothrombin binding occurs via the RGD sequence in this proenzyme and found that RGD-containing peptides blocked this interaction. Since GP IIb–IIIa binding enhanced the rate of prothrombin activation, a reaction that was blocked by GP IIb–IIIa antagonists, these data suggest that GP IIb–IIIa antagonists may also inhibit clot formation because they displace prothrombin binding to the platelet surface. Either explanation could account for

Cambridge Books Online © Cambridge University Press, 2009

the long clotting times in the platelet rich plasma (PRP) from patients with Glanzmann's thrombasthenia[90].

Fibrinolysis

Recent studies suggest that GP IIb–IIIa antagonists may augment the ability of fibrinolytics to achieve patency in occluded arteries[91,92]. As shown in Fig. 63.2(*c*), there are two primary mechanistic reasons why GP IIb–IIIa antagonists have this activity. First, platelet stimuli are generated by thrombolysis. This can occur by plasmin, known to have a platelet stimulatory activity[93–95], thrombin released from clots during lysis[96] or the adhesive proteins on the vessel wall re-expressed during thrombolysis. GP IIb–IIIa antagonists prevent platelet recruitment by these stimuli. Secondly, platelet alpha granules contain PAI-1 (plasminogen activator inhibitor) which is secreted during platelet thrombosis. GP IIb–IIIa antagonists block platelet recruitment and the secretion of this negative modulator of fibrinolysis. Ongoing clinical trials are designed to establish conditions and protocols for the efficacious combination of GP IIb–IIIa antagonists with thrombolytics[97].

Inflammation

Atherosclerotic cardiovascular disease is the consequence of vascular inflammation[98]. The fibrinogen binding activity of GP IIb–IIIa may contribute to this process in four ways. First, fibrinogen bound to GP IIb–IIIa can bind to Mac-1, a $\beta 2$ integrin, and recruit neutrophils into a growing thrombus[99]. Second, bound fibrinogen, together with aggregation, induces outside-in GP IIb–IIIa signalling and alpha granule secretion resulting in the release of inflammatory mediators such as TGFβ, PF4, RANTES and other chemokines[100,101]. Third, outside-in GP IIb–IIIa signalling also enhances the production of inflammatory mediators from the coagulation cascade such as Factor Xa[102]. Perhaps most importantly, fibrinogen bound to GP IIb–IIIa also mediates the binding of platelets to endothelial cells and leukocytes so that the CD40 ligand (CD40L) expressed on the platelet surface can induce an inflammatory response through these cells[103]. CD40L (CD154, gp 39), is a membrane protein in the TNF family and is a potent proinflammatory factor originally identified in $CD4^+$ T lymphocytes. The platelet location was established in a pioneering study by Henn et al.[103], who showed that while CD40L was not exposed on the surface of unstimulated, discoid platelets, it rapidly became exposed on platelets during thrombin-induced platelet activation. CD40L is a ligand for CD40, an inflammation receptor found on many vascular cells and, indeed, incubation of aggregated platelets that bear exposed CD40L with vascular cells induced an inflammatory response as detected by the release of inflammatory cytokines and the expression of adhesion receptors, e.g. ICAM-1, VCAM-1, and tissue factor[104]. A critical role for CD40L in atherosclerosis has been established by studies showing that CD40L antibodies[105] or CD40L gene targeting[106] reduce atherosclerotic lesion development in mice prone to atherosclerosis. Aukrust et al.[107], showed that a soluble form of CD40L was generated in patients with acute coronary thrombotic syndromes, e.g. unstable angina and with complications of PCI and that the soluble CD40L was inflammatory to peripheral blood mononuclear cells. Since aggregated platelets are associated with atherosclerosis throughout lesion progression and since the adhesion of platelets to the CD40L-responsive cells within the vasculature is mediated by GP IIb–IIIa, it follows that GP IIb–IIIa antagonism may inhibit the CD40L inflammatory activity. An untested hypothesis is that GP IIb–IIIa antagonists may reduce the pathological consequences of inflammation and atherosclerotic lesion progression in acute coronary syndromes.

Orally available GP IIb–IIIa antagonists

Because of the clinical benefits demonstrated by the three parenterally available GP IIb–IIIa antagonists and the hope of extending the clinical benefits by GP IIb–IIIa antagonism beyond the in-hospital administration of therapy, extensive effort has been directed towards the development of orally available GP IIb–IIIa antagonists. Most of these antagonists are mono- or double-prodrugs that are rapidly converted to their active forms in circulation. Although these antagonists are of diverse chemical structures, the active forms of each have a common structural feature, positive and negative charges separated by a distance of 10–20 angstroms[108], a distance is similar to that between the positive charge of arginine and the negative charge of aspartic acid on the Arg–Gly–Asp (RGD) sequence, and a sequence responsible for the GP IIb–IIIa binding activities of several adhesive proteins including vWf, vitronectin and fibronectin[109].

As of writing, five placebo controlled phase III clinical trials have been performed using the orally available GP IIb–IIIa antagonists. None have shown a clinical benefit. The clinical conditions evaluated are diverse and include unstable coronary syndrome, percutaneous revascularization and peripheral vascular disease. Because of the lack of clinical success, the development of the drugs has either been terminated, or is on hold. Several authors have discussed possible reasons why the orally available GP

Cambridge Books Online © Cambridge University Press, 2009

IIb–IIIa antagonists have not yet shown clinical benefit. Adderley and Fitzgerald[110] found in in vitro studies that orbofiban and xemilofiban induced dose-dependent apoptosis and procaspase-3 activation in cardiomyocytes, suggesting that failure of these drugs in the clinic could have been due to their induction of cell death. However, since these studies were performed with the proforms of the antagonists, forms that have low concentrations in circulation, the apoptotic hypothesis needs to be supported by additional studies using the active forms of these drugs, at concentrations closer to that used in the clinic, which also induce an apoptotic response. Another possibility is that the pharmacokinetic profiles of the tested drugs, which permits wide variations in the extent of platelet coverage during the 8-to-12 hours between dosing[111], allows for extended periods where insufficient GP IIb–IIIa antagonism is achieved[112]. This undoubtedly occurred. Presumably, orally available antagonists with improved pharmacokinetic profiles, possibly achieved by once a day formulations with little change in plasma levels during this time interval, may solve this problem. A third possibility is the lack of aspirin in the treatment arm (e.g. Symphony I using sibrafiban[113]) or non-compliance of aspirin use during other trials. Aspirin has been found to synergize with GP IIb–IIIa antagonists in blocking aggregation to collagen[114]. Since collagen is an integral component in atheromas and would be expected to be involved in platelet stimulation following plaque rupture[115], less than optimal inhibition of platelet aggregation to this agonist, as would be provided by the noninclusion of aspirin, may have contributed to a less than optimal therapeutic outcome. A fourth possibility is that the suboptimal doses used chronically, i.e. 10–60% inhibition of platelet aggregation, may provide negative consequences such as the enhancement of inflammation or thrombosis. One example is CD40L, an inflammatory protein that is known to be involved in the progression of atherosclerosis. GP IIb–IIIa antagonists both positively and negatively regulate the release CD40L in vitro: optimal concentrations inhibit, e.g. doses that achieve >60% inhibition of platelet aggregation; surprisingly, suboptimal concentrations potentiate release, e.g. doses that achieve 20–50% inhibition of platelet aggregation (L. Nannizzi-Alaimo, S. Prasad and D. Phillips, unpublished observations). These findings predict that suboptimal doses of the orally available GP IIb–IIIa antagonists may have caused an enhancement of inflammation. Although experiments have been performed which suggest that selected orally available GP IIb–IIIa antagonists may potentiate platelet stimulation, indicating that they may also paradoxically potentiate thrombosis[116], it remains to be determined whether this occurs during the clinical use of these agents. These considerations predict that an orally available GP IIb–IIIa antagonist with an improved pharmacokinetic profile such as demonstrated by Roxifiban[117], or other GP IIb–IIIa under development, may have a therapeutic benefit not achieved in previous clinical trials of this class of drugs.

Conclusions

The combined study of Glanzmann's thrombasthenia, of GP IIb–IIIa, and of GP IIb–IIIa antagonists in arterial thrombotic disease have now clearly established the pivotal roles of GP IIb–IIIa in platelet aggregation and of platelet aggregation in arterial thrombosis. Use of GP IIb–IIIa antagonists is now a proven strategy for the reduction of arterial thrombosis to reduce morbidity and mortality in human disease. It is now established that aggregation is only part of GP IIb–IIIa function in that its signalling in platelets facilitates a wide range of reactions such as platelet shape change, secretion of alpha granule and dense body constituents, consolidation of aggregates and clot retraction. These functions of GP IIb–IIIa indicate that it is not only involved in thrombosis and hemostasis but also links platelets and platelet aggregates to coagulation, inflammation, thrombolysis, atherosclerosis and the proliferation of cells in the vessel wall in a dynamic way, more than would result from simple vessel occlusion. Thus, it is predicted that GP IIb–IIIa antagonists may provide additional benefits beyond simple prevention of vessel occlusion. Data are also accumulating that GP IIb–IIIa antagonists also provide added benefit in combination with other antithrombotics and with fibrinolytics. All initial trials were performed in combination with aspirin and heparin: data are emerging on combinations with clopidigrel (Plavix®) and low molecular weight heparins. Data are also available suggesting that GP IIb–IIIa antagonists act synergistically with fibrinolytics in the setting of acute myocardial infarction, suggesting that such combinations may be a preferred therapy. Future clinical trials are required to evaluate these possibilities.

Given the pivotal role of GP IIb–IIIa in platelet function, it has been disappointing that orally available GP IIb–IIIa antagonists have not yet provided a therapeutic benefit. However, newer orally available GP IIb–IIIa antagonists are now available with improved pharmacokinetic and pharmacodynamic profiles, and it may well be of great interest to understand how these agents perform in large-scale clinical trials. It is also possible that alternative strategies might be developed to regulate GP IIb–IIIa function. For example, future directions on GP IIb–IIIa research can be

Cambridge Books Online © Cambridge University Press, 2009

expected to continue to focus on extracellular and intracellular domains, providing new insights into the molecular description of how GP IIb–IIIa interacts with its ligands and into how GP IIb–IIIa is involved in the various signal transduction pathways. Evidence, both from naturally occurring and experimentally induced mutations of GP IIb–IIIa, clearly indicate a role for the cytoplasmic domains of GP IIb and GP IIIa in the function of GP IIb–IIIa, both in linking the integrin to the platelet cytoskeleton and in associations with signalling proteins. These proximal events in GP IIb–IIIa signalling may offer new targets for pharmacological intervention of GP IIb–IIIa which may provide for inhibition of platelet aggregation and arterial thrombosis with minimal effects on hemostasis.

References

1 Hynes RO. Integrins: versatility, modulation, and signalling in cell adhesion. *Cell* 1992; 69: 11.

2 Coller BS. Platelet GPIIb/IIIa antagonists: the first anti-integrin receptor therapeutics. *J Clin Invest* 1997; 99: 1467–1471.

3 Lefkovits J, Topol EJ. The clinical role of platelet glycoprotein IIb/IIIa receptor inhibitors in ischemic heart disease. *Cleve Clin J Med* 1996; 63: 181–189.

4 Phillips DR, Scarborough RM. Clinical pharmacology of eptifibatide. *Am J Cardiol* 1997; 80: 11B–20B.

5 Bhatt DL, Topol EJ. Current role of platelet glycoprotein IIb/IIIa inhibitors in acute coronary syndromes. *JAMA* 2000; 284: 1549–1558.

6 Topol EJ, Byzova TV, Plow EF. Platelet GPIIb–IIIa blockers. *Lancet* 1999; 353: 227–231.

7 Wagner CL, Mascelli, MA, Neblock, DS, Weisman, HF, Coller BS, Jordan RE. Analysis of GP IIb/IIIa receptor number by quantification of 7E3 binding to human platelets. *Blood* 1996; 88: 907–914.

8 Youssefian T, Masse JM, Rendu F, Guichard J, Cramer EM. Platelet and megakaryocyte dense granules contain glycoproteins Ib and IIb–IIIa. *Blood* 1997; 89: 4047–4057.

9 Shattil S, Kashiwagi H, Pampori N. Integrin signalling: the platelet paradigm. *Blood* 1998; 91: 1–14.

10 Wencel-Drake JD, Plow EF, Kunicki TJ, Woods VL, Keller DM, Ginsberg MH. Localization of internal pools of membrane glycoproteins involved in platelet adhesive responses. *Am J Pathol* 1986; 124: 324–334.

11 Handagama P, Scarborough RM, Shuman MA, Bainton DF. Endocytosis of fibrinogen into megakaryocyte and platelet alpha- granules is mediated by alpha IIb beta 3 (glycoprotein IIb–IIIa). *Blood* 1993; 82: 135–138.

12 Wencel-Drake JD, Frelinger ALD, Dieter MG, Lam SC. Arg–Gly–Asp-dependent occupancy of GPIIb/IIIa by applaggin: evidence for internalization and cycling of a platelet integrin. *Blood* 1993; 81: 62–69.

13 Nurden P, Poujol C, Durrieu-Jais C et al. Labeling of the internal pool of GP IIb–IIIa in platelets by c7E3 Fab fragments (abciximab): flow and endocytic mechanisms contribute to the transport. *Blood* 1999; 93: 1622–1633.

14 Ni H, Denis CV, Subbarao S et al. Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. *J Clin Invest* 2000; 106: 385–392.

15 Shiba E, Lindon JN, Kushner L et al. Antibody-detectable changes in fibrinogen adsorption affecting platelet activation on polymer surfaces. *Am J Physiol* 1991; 260: C965–C974.

16 Gawaz MP, Loftus JC, Bajt ML, Frojmovic MM, Plow EF, Ginsberg MH. Ligand bridging mediates integrin alpha IIb beta 3 (platelet GPIIB–IIIA) dependent homotypic and heterotypic cell–cell interactions. *J Clin Invest* 1991; 88: 1128–1134.

17 Pratico D, Cyrus T, Li H, FitzGerald GA. Endogenous biosynthesis of thromboxane and prostacyclin in 2 distinct murine models of atherosclerosis. *Blood* 2000; 96: 3823–3826.

18 Basse F, Stout JG, Sims PJ, Wiedmer T. Isolation of an erythrocyte membrane protein that mediates Ca2+- dependent transbilayer movement of phospholipid. *J Biol Chem* 1996; 271: 17205–17210.

19 Smeets EF, Comfurius P, Bevers EM, Zwaal RF. Calcium-induced transbilayer scrambling of fluorescent phospholipid analogs in platelets and erythrocytes. *Biochim Biophys Acta* 1994; 1195: 281–286.

20 Sims PJ, Wiedmer T, Esmon CT, Weiss HJ, Shattil SJ. Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome: an isolated defect in platelet procoagulant activity. *J Biol Chem* 1989; 264: 17049–17057.

21 Phillips DR, Charo IF, Parise LV, Fitzgerald LA. The platelet membrane glycoprotein IIb–IIIa complex. *Blood* 1988; 71: 831–843.

22 Law DA, Nannizzi-Alaimo L, Phillips DR. Outside-in integrin signal transduction. Alpha IIb beta 3–(GP IIb IIIa) tyrosine phosphorylation induced by platelet aggregation. *J Biol Chem* 1996; 271: 10811–10815.

23 Jenkins A, Nannizzi-Alaimo L, Silver D, Sellers J et al. Tyrosine phosphorylation of the b3 cytoplasmic domain mediates integrin–cytoskeletal interactions. 1998: Submitted for publication.

24 Cowan KJ, Law DA, Phillips DR. Identification of shc as the primary protein binding to the tyrosine- phosphorylated beta 3 subunit of alpha IIbbeta 3 during outside-in integrin platelet signalling. *J Biol Chem* 2000; 275: 36423–36429.

25 Law DA, Nannizzi-Alaimo L, Cowan KJ, Prasad KS, Ramakrishnan V, Phillips DR. Signal transduction pathways for mouse platelet membrane adhesion receptors. *Thromb Haemost* 1999; 82: 345.

26 Hollopeter G, Jantzen HM, Vincent D et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* 2001; 409: 202–207.

27 Kamiguti AS, Theakston RD, Watson SP, Bon C, Laing GD,

Cambridge Books Online © Cambridge University Press, 2009

Zuzel M. Distinct contributions of glycoprotein VI and alpha(2)beta(1) integrin to the induction of platelet protein tyrosine phosphorylation and aggregation. *Arch Biochem Biophys* 2000; 374: 356–362.

28 Keely PJ, Parise LV. The alpha2beta1 integrin is a necessary co-receptor for collagen- induced activation of Syk and the subsequent phosphorylation of phospholipase Cgamma2 in platelets. *J Biol Chem* 1996; 271: 26668–26676.

29 Coughlin SR. Thrombin signalling and protease-activated receptors. *Nature* 2000; 407: 258.

30 Ramakrishnan V, DeGuzman F, Bao M, Hall SW, Leung LL, Phillips DR. A thrombin receptor function for platelet glycoprotein Ib-IX unmasked by cleavage of glycoprotein V. *Proc Natl Acad Sci USA* 2001; 98: 1823–1828.

31 Andrews R, Harris S, McNally T, Berndt M. Binding of the purified 14–3–3z signalling protein to discrete amino acid sequences within the cytoplasmic domain of the platelet membrane glycoprotein Ib-IX-V complex. *Biochemistry* 1998; 37: 638–647.

32 Coller BS. Platelets and thrombolytic therapy. *N Engl J Med* 1990; 322: 33–42.

33 Weisman HF. Anti-platelet monoclonal antibodies for the prevention of arterial thrombosis: experience with ReoPro, a monoclonal antibody directed against the platelet GPIIb/IIIa receptor. *Biochem Soc Trans* 1995; 23: 1051–1057.

34 Lynch JJ, Jr, Cook JJ, Sitko GR et al. Nonpeptide glycoprotein IIb/IIIa inhibitors. 5. Antithrombotic effects of MK-0383. *J Pharmacol Exp Ther* 1995; 272: 20–32.

35 Scarborough RM. Development of eptifibatide. *Am Heart J* 1999; 138: 1093–1104.

36 Braunwald E, Antman EM, Beasley JW et al. ACC/AHA guidelines for the management of patients with unstable angina and non-ST-segment elevation myocardial infarction. A report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee on the Management of Patients With Unstable Angina). *J Am Coll Cardiol* 2000; 36: 970–1062.

37 Lincoff AM, Califf RM, Topol EJ. Platelet glycoprotein IIb/IIIa receptor blockade in coronary artery disease. *J Am Coll Cardiol* 2000; 35: 1103–1115.

38 Scarborough RM, Kleiman NS, Phillips DR. Platelet glycoprotein IIb/IIIa antagonists. What are the relevant issues concerning their pharmacology and clinical use? *Circulation* 1999; 100: 437–444.

39 Kereiakes DJ, Broderick TM, Roth EM et al. Time course, magnitude, and consistency of platelet inhibition by abciximab, tirofiban, or eptifibatide in patients with unstable angina pectoris undergoing percutaneous coronary intervention. *Am J Cardiol* 1999; 84: 391–395.

40 Kereiakes DJ, Broderick TM, Roth EM, Whang D, Mueller M, Lacock P et al. High platelet count in platelet-rich plasma reduces measured platelet inhibition by abciximab but not tirofiban nor eptifibatide glycoprotein IIb/IIIa receptor antagonists. *J Thromb Thrombolysis* 2000; 9: 149–155.

41 Peter K, Kohler B, Straub A et al. Flow cytometric monitoring of glycoprotein IIb/IIIa blockade and platelet function in patients with acute myocardial infarction receiving reteplase, abciximab, and ticlopidine: continuous platelet inhibition by the combination of abciximab and ticlopidine. *Circulation* 2000; 102: 1490–1496.

42 Abulencia JP, Tien N, McCarty OJ, Plymire D, Mousa SA, Konstantopoulos K. Comparative antiplatelet efficacy of a novel, nonpeptide GPIIb/IIIa antagonist (XV454) and abciximab (c7E3) in flow models of thrombosis. *Arterioscler Thromb Vasc Biol* 2001; 21: 149–156.

43 Cierniewski CS, Haas TA, Smith JW, Plow EF. Characterization of cation-binding sequences in the platelet integrin GPIIb–IIIa (alpha IIb beta 3) by terbium luminescence. *Biochemistry* 1994; 33: 12238–12246.

44 Marguerie GA, Edgington TS, Plow EF. Interaction of fibrinogen with its platelet receptor as part of a multistep reaction in ADP-induced platelet aggregation. *J Biol Chem* 1980; 255: 154–161.

45 Phillips DR, Teng W, Arfsten A et al. Effect of Ca^{2+} on GP IIb–IIIa interactions with integrilin: enhanced GP IIb–IIIa binding and inhibition of platelet aggregation by reductions in the concentration of ionized calcium in plasma anticoagulated with citrate [In Process Citation]. *Circulation* 1997; 96: 1488–1494.

46 Tcheng JE, Harrington RA, Kottke-Marchant K et al. Multicenter, randomized, double-blind, placebo-controlled trial of the platelet integrin glycoprotein IIb/IIIa blocker Integrelin in elective coronary intervention. IMPACT Investigators. *Circulation* 1995; 91: 2151–2157.

47 Inhibition of platelet glycoprotein IIb/IIIa with eptifibatide in patients with acute coronary syndromes. The PURSUIT Trial Investigators. Platelet glycoprotein IIb/IIIa in unstable angina: receptor suppression using integrilin therapy. *N Engl J Med* 1998; 339: 436–443.

48 Novel dosing regimen of eptifibatide in planned coronary stent implantation (ESPRIT): a randomised, placebo-controlled trial. *Lancet* 2000; 356: 2037–2044.

49 Marciniak JSJ, Jordan, RE, Masceli, MA. Effect of Ca^{2+} chelation on the platelet inhibitory ability of the GPIIb/IIIa antagonists Abciximab, Eptifibatide and Tirofiban. *Thromb Haemost* 2001; 85: 539–542.

50 Gralnick HR, Williams S, McKeown L et al. Endogenous platelet fibrinogen surface expression on activated platelets. *J Lab Clin Med* 1991; 118: 604–613.

51 Legrand C, Dubernard V, Nurden AT. Studies on the mechanism of expression of secreted fibrinogen on the surface of activated human platelets. *Blood* 1989; 73: 1226–1234.

52 Woods VL, Wolff LE, Keller DM. Resting platelets contain a substantial centrally located pool of glycoprotein IIb–IIIa complex which may be accessible to some but not other extracellular proteins. *J Biol Chem* 1986; 261: 15242–15251.

53 Bihour C, Durrieu-Jais C, Macchi L et al. Expression of markers of platelet activation and the interpatient variation in response to abciximab. *Arterioscler Thromb Vasc Biol* 1999; 19: 212–219.

Cambridge Books Online © Cambridge University Press, 2009

54 Gawaz M, Ruf A, Pogatsa-Murray G et al. Incomplete inhibition of platelet aggregation and glycoprotein IIb–IIIa receptor blockade by abciximab: importance of internal pool of glycoprotein IIb–IIIa receptors. *Thromb Haemost* 2000; 83: 915–922.
55 Hartwell DW, Wagner DD. New discoveries with mice mutant in endothelial and platelet selectins. *Thromb Haemost* 1999; 82: 850–857.
56 Neumann FJ, Hochholzer W, Pogatsa-Murray G, Schomig A, Gawaz M. Antiplatelet effects of abciximab, tirofiban and eptifibatide in patients undergoing coronary stenting. *J Am Coll Cardiol* 2001; 37: 1323–1328.
57 Phillips DR, Teng W, Arfsten A et al. Effect of Ca^{2+} on GP IIb–IIIa interactions with integrilin: enhanced GP IIb–IIIa binding and inhibition of platelet aggregation by reductions in the concentration of ionized calcium in plasma anticoagulated with citrate. *Circulation* 1997; 96: 1488–1494.
58 Cook JJ, Glass JD, Sitko GR et al. Nonpeptide glycoprotein IIb/IIIa inhibitors: 14: oral antithrombotic efficacy of L-738,167 in a conscious canine model of coronary artery electrolytic injury. *Circulation* 1997; 96: 949–958.
59 Mukherjee D, Moliterno DJ. Monitoring antiplatelet therapy: what is the best method? *Clin Pharmacokinet* 2000; 39: 445–458.
60 Osende JI, Fuster V, Lev EI et al. Testing platelet activation with a shear-dependent platelet function test versus aggregation-based tests: relevance for monitoring long-term glycoprotein iib/iiia inhibition. *Circulation* 2001; 103: 1488–1491.
61 Kereiakes DJ, Runyon JP, Broderick TM, Shimshak TM. IIbs are not IIbs. *Am J Cardiol* 2000; 85: 23C–31C.
62 Hoshiga M, Alpers CE, Smith LL, Giachelli CM, Schwartz SM. Alpha-v beta-3 integrin expression in normal and atherosclerotic artery. *Circ Res* 1995; 77: 1129–1135.
63 Coleman KR, Braden GA, Willingham MC, Sane DC. Vitaxin, a humanized monoclonal antibody to the vitronectin receptor (alphavbeta3), reduces neointimal hyperplasia and total vessel area after balloon injury in hypercholesterolemic rabbits. *Circ Res* 1999; 84: 1268–1276.
64 Srivatsa SS, Fitzpatrick LA, Tsao PW et al. Selective alpha v beta 3 integrin blockade potently limits neointimal hyperplasia and lumen stenosis following deep coronary arterial stent injury: evidence for the functional importance of integrin alpha v beta 3 and osteopontin expression during neointima formation. *Cardiovasc Res* 1997; 36: 408–428.
65 Neumann FJ, Kastrati A, Schmitt C et al. Effect of glycoprotein IIb/IIIa receptor blockade with abciximab on clinical and angiographic restenosis rate after the placement of coronary stents following acute myocardial infarction. *J Am Coll Cardiol* 2000; 35: 915–921.
66 Deitch JS, Williams JK, Adams MR et al. Effects of beta3-integrin blockade (c7E3) on the response to angioplasty and intra-arterial stenting in atherosclerotic nonhuman primates. *Arterioscler Thromb Vasc Biol* 1998; 18: 1730–1737.
67 Ikari Y, Yee KO, Schwartz SM. Role of alpha5beta1 and alphavbeta3 integrins on smooth muscle cell spreading and migration in fibrin gels. *Thromb Haemost* 2000; 84: 701–705.
68 Simon DI, Dhen Z, Seifert P, Edelman ER, Ballantyne CM, Rogers C. Decreased neointimal formation in Mac-1(−/−) mice reveals a role for inflammation in vascular repair after angioplasty. *J Clin Invest* 2000; 105: 293–300.
69 Diamond MS, Garcia-Aguilar J, Bickford JK, Corbi AL, Springer TA. The I domain is a major recognition site on the leukocyte integrin Mac- 1 (CD11b/CD18) for four distinct adhesion ligands. *J Cell Biol* 1993; 120: 1031–1043.
70 Altieri DC, Edgington TS. A monoclonal antibody reacting with distinct adhesion molecules defines a transition in the functional state of the receptor CD11b/CD18 (Mac-1). *J Immunol* 1988; 141: 2656–2660.
71 Mickelson JK, Ali MN, Kleiman NS et al. Chimeric 7E3 Fab (ReoPro) decreases detectable CD11b on neutrophils from patients undergoing coronary angioplasty. *J Am Coll Cardiol* 1999; 33: 97–106.
72 Kouns WC, Kirchhofer D, Hadvary P et al. Reversible conformational changes induced in glycoprotein IIb–IIIa by a potent and selective peptidomimetic inhibitor. *Blood* 1992; 80: 2539–2547.
73 Jennings LK, Haga JH, Slack SM. Differential expression of a ligand induced binding site (LIBS) by GPIIb–IIIa ligand recognition peptides and parenteral antagonists. *Thromb Haemost* 2000; 84: 1095.
74 McQueney P, Bollag D, Egbertson M, Hartmen G, Gould R, Bednar B. RGD ligands induce a common neo-epitpoe upon binding to aIIbb3 on platelets. *Mol Biol Cell* 1996; 7: 247a [abstract 1436].
75 Dickfeld T, Ruf A, Pogasta-Murray G et al. Differential antiplatelet effects of various glycoprotein IIb–IIIa antagonists. *Thrombosis Res* 2001; 101: 53–64.
76 Ferrari E, Thiry M, Touati C, Gibelin P, Baudouy M. Acute profound thrombocytopenia after c7E3 Fab therapy. *Circulation* 1997; 96: 3809–3810.
77 Simpfendorfer C, Kottke-Marchant K, Lowrie M et al. First chronic platelet glycoprotein IIb/IIIa integrin blockade. A randomized, placebo-controlled pilot study of Xemlifoban in unstable angina with percutaneous coronary interventions. *Circulation* 1997; 96: 76–81.
78 Cannon C, McCabe C, Borzak S et al. Randomized trial of an oral platelet glycoprotein IIb/IIIa antagonist, sibrafiban, in patients after an acute coronary syndrome. *Circulation* 1998; 97: 340–349.
79 Bednar B, Bednar R, Cook J et al. Drug-dependent antibodies against GPIIb/IIIa induce thrombocytopenia. *Circulation* 1996; 94: 1–99.
80 Giugliano RP. Drug-induced thrombocytopenia: is it a serious concern for glycoprotein IIb/IIIa receptor inhibitors? *J Thromb Thrombolysis* 1998; 5: 191–202.
81 Dasgupta H, Blankenship JC, Wood GC, Frey CM, Demko SL, Menapace FJ. Thrombocytopenia complicating treatment with intravenous glycoprotein IIb/IIIa receptor inhibitors: a pooled analysis. *Am Heart J* 2000; 140: 206–211.
82 Reverter J, Beguin S, Kessels R, Hemker H, Coller B. Inhibition of platelet-mediated, tissue factor-induced thrombin gener-

Cambridge Books Online © Cambridge University Press, 2009

ation by the mouse/human chimeric 7E3 antibody. *J Clin Invest* 1996; 98: 863–874.
83 Herault J, Peyrou V, Savi P, Bernat A, Herbert J. Effect of SR121566A, a potent GP IIb–IIIa antagonist on platelet-mediated thrombin generation in vitro and in vivo. *Thromb Haemost* 1998; 79: 383.
84 Butenas S, Cawthern KM, van't Veer C, DiLorenzo ME, Lock JB, Mann KG. Antiplatelet agents in tissue factor-induced blood coagulation. *Blood* 2001; 97: 2314–2322.
85 Pedicord DL, Thomas BE, Mousa SA, Dicker IB. Glycoprotein IIb/IIIa receptor antagonists inhibit the development of platelet procoagulant activity. *Thromb Res* 1998; 90: 247–258.
86 Moliterno DJ, Califf RM, Aguirre FV et al. Effect of platelet glycoprotein IIb/IIIa integrin blockade on activated clotting time during percutaneous transluminal coronary angioplasty or directional atherectomy (the EPIC trial). Evaluation of c7E3 Fab in the Prevention of Ischemic Complications trial. *Am J Cardiol* 1995; 75: 559–562.
87 Swords N, Tracy P, Mann K. Intact platelet membranes, not platelet-released microvesicles, support the procoagulant activity of adherent platelets. *Arterioscler Thromb* 1993; 13: 1613–1622.
88 Li Y, Spencer FA, Ball S, Becker RC. Inhibition of platelet-dependent prothrombinase activity and thrombin generation by glycoprotein IIb/IIIa receptor-directed antagonists: potential contributing mechanism of benefit in acute coronary syndromes. *J Thromb Thrombolysis* 2000; 10: 69–76.
89 Byzova TV, Plow EF. Networking in the hemostatic system. *J Biol Chem* 1997; 272: 27183–27188.
90 Basic-Micic M, Roman C, Herpel U, Kling E, Scholz B, Breddin H. Platelet-induced thrombin generation time: a new sensitive global assay for platelet function and coagulation. Method and first results. *Haemostasis* 1992; 22: 309–321.
91 Campbell KR, Ohman EM, Cantor W, Lincoff AM. The use of glycoprotein IIb/IIIa inhibitor therapy in acute ST-segment elevation myocardial infarction: current practice and future trends. *Am J Cardiol* 2000; 85: 32C–38C.
92 Cannon CP. Learning from the recently completed oral glycoprotein IIb/IIIa receptor antagonist trials. *Clin Cardiol* 2000; 23: VI-14–17.
93 Kinlough-Rathbone RL, Perry DW, Rand ML, Packham MA. Pretreatment of human platelets with plasmin inhibits responses to thrombin, but potentiates responses to low concentrations of aggregating agents, including the thrombin receptor activating peptide, SFLLRN. *Thromb Haemost* 1997; 77: 741–747.
94 Rabhi-Sabile S, Pidard D. Exposure of human platelets to plasmin results in the expression of irreversibly active fibrinogen receptors. *Thromb Haemost* 1995; 73: 693–701.
95 Schafer AI, Maas AK, Ware JA, Johnson PC, Rittenhouse SE, Salzman EW. Platelet protein phosphorylation, elevation of cytosolic calcium, and inositol phospholipid breakdown in platelet activation induced by plasmin. *J Clin Invest* 1986; 78: 73–79.
96 Weitz JI, Hudoba M, Massel D, Maraganore J, Hirsh J. Clot-bound thrombin is protected from inhibition by heparin-antithrombin III but is susceptible to inactivation by anti-thrombin III- independent inhibitors. *J Clin Invest* 1990; 86: 385–391.
97 Helft G, Worthley SG, Zaman AG, Samama MM, Badimon JJ. Thrombolysis and adjunctive therapies in acute myocardial infarction. *Haemostasis* 2000; 30: 159–167.
98 Ross R. Atherosclerosis – an inflammatory disease. *N Engl J Med* 1999; 340: 115–126.
99 Weber C, Springer T. Neutrophil accumulation on activated, surface-adherent platelets in flow is mediated by interaction of Mac-1 with fibrinogen bound to aIIbb3 and stimulated by platelet-activating factor. *J Clin Invest* 1997; 100: 2085–2093.
100 Schini-Kerth V, Bassus S, Fisslthaler B, Kirchmaier C, Busse R. Aggregating human platelets stimulate the expression of thrombin receptors in cultured vascular smooth muscle cells via the release of transforming growth factor-beta-1 and platelet-derived growth factor AB. *Circulation* 1997; 96: 3888–3896.
101 Power CA, Clemetson JM, Clemetson KJ, Wells TN. Chemokine and chemokine receptor mRNA expression in human platelets. *Cytokine* 1995; 7: 479–482.
102 Gillis S, Furie B, Furie B. Interactions of neutrophils and coagulation proteins. *Semin Hemat* 1997; 34: 336–342.
103 Henn V, Slupsky J, Grafe M et al. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 1998; 391: 591.
104 Mach F, Schonbeck U, Bonnefoy J-Y, Pober J, Libby P. Activation of monocyte/macrophage functions related to acute atheroma complication by ligation of CD40. *Circulation* 1997; 96: 396–399.
105 Mach F, Schonbeck U, Sukhova GK, Atkinson E, Libby P. Reduction of atherosclerosis in mice by inhibition of CD40 signalling. *Nature* 1998; 394: 200–203.
106 Lutgens E, Gorelik L, Daemen MJ et al. Requirement for CD154 in the progression of atherosclerosis. *Nat Med* 1999; 5: 1313–1316.
107 Aukrust P, Muller F, Ueland T et al. Enhanced levels of soluble and membrane-bound CD40 ligand in patients with unstable angina. Possible reflection of T lymphocyte and platelet involvement in the pathogenesis of acute coronary syndromes. *Circulation* 1999; 100: 614–620.
108 Hartman GD, Egbertson MS, Halczenko W et al. Non-peptide fibrinogen receptor antagonists. 1. Discovery and design of exosite inhibitors. *J Med Chem* 1992; 35: 4640–4642.
109 Zablocki JA, Miyano M, Rao SN, Panzer-Knodle S, Nicholson N, Feigen L. Potent inhibitors of platelet aggregation based upon the Arg–Gly–Asp–Phe sequence of fibrinogen. A proposal on the nature of the binding interaction between the Asp–carboxylate of RGDX mimetics and the platelet GP IIb–IIIa receptor. *J Med Chem* 1992; 35: 4914–4917.
110 Adderley SR, Fitzgerald DJ. Glycoprotein IIb/IIIa antagonists induce apoptosis in rat cardiomyocytes by caspase-3 activation. *J Biol Chem* 2000; 275: 5760–5766.

Cambridge Books Online © Cambridge University Press, 2009

111 Quinn MJ, Cox D, Foley JB, Fitzgerald DJ. Glycoprotein IIb/IIIa receptor number and occupancy during chronic administration of an oral antagonist. *J Pharmacol Exp Ther* 2000; 295: 670–676.
112 Reimann JD, Modi NB, Novotny W. Pharmacokinetics and pharmacodynamics of sibrafiban, an orally administered GP IIb/IIIa antagonist, following coadministration of aspirin and heparin. *J Clin Pharmacol* 2000; 40: 488–495.
113 Comparison of sibrafiban with aspirin for prevention of cardiovascular events after acute coronary syndromes: a randomised trial. The SYMPHONY Investigators. Sibrafiban versus aspirin to yield maximum protection from ischemic heart events post-acute coronary syndromes. *Lancet* 2000; 355: 337–345.
114 Frederick LG, Suleymanov OD, Szalony JA et al. Protective effect of oral xemilofiban in arterial thrombosis in dogs: increased activity in combination with aspirin. *Circulation* 1998; 98: 813–820.
115 Mann JM, Davies MJ. Vulnerable plaque. Relation of characteristics to degree of stenosis in human coronary arteries. *Circulation* 1996; 94: 928–931.
116 Honda S, Tomiyama Y, Aoki T et al. Association between ligand-induced conformational changes of integrin IIbbeta3 and IIbbeta3-mediated intracellular Ca^{2+} signalling. *Blood* 1998; 92: 3675–3683.
117 Mousa SA, Bozarth JM, Forsythe MS, Slee A. Differential antiplatelet efficacy for various GPIIb/IIIa antagonists: role of plasma calcium levels. *Cardiovasc Res* 2000; 47: 819–826.

Cambridge Books Online © Cambridge University Press, 2009

Other antiplatelet agents

Luigi Iuliano, Fausta Micheletta and Francesco Violi

University of Rome La Sapienza, Institute of Clinical Medicine I, Rome, Italy

Reversible COX-1 inhibitors

While aspirin induces irreversible acetylation of the cyclooxygenase moiety of the PGG/H synthase enzyme, other non-steroidal anti-inflammatory drugs (NSAIDs) bind reversibly at, or close to, the active site.

Several NSAIDs have been tested as inhibitors of platelet function. Their effects are reversible within several hours, necessitating multiple daily doses to produce important inhibition of thromboxane A_2[1]. The use of NSAID is also limited by the high rate of gastrointestinal side effects. During naproxen therapy, 400 mg twice a day, increasing the bleeding time, the rate of endoscopically determined gastroduodenal ulcers was 26%, significantly greater than with placebo[2]. The prevalence of gastroduodenal ulcers is from 15% to 30% among users of other NSAIDs[3–5].

Sulfinpyrazone is one of the most studied platelet COX inhibitors. The inhibitory effects of sulfinpyrazone on platelets are more marked ex vivo than in vitro. This behaviour is shared with other antiplatelet drugs including ticlopidine and is dependent on active metabolites formed in vivo. The thioether metabolite of sulfinpyrazone is between 8 and 13 times more potent than the parent compound as an inhibitor of platelet aggregation induced by arachidonic acid[6]. Platelet inhibition is transient with sulfinpyrazone and, after several daily regimens of 200 mg four times a day, inhibition of platelet aggregation no longer is evident 24 hours after the final dose[7]. Initial results from clinical trials using sulfinpyrazone in the secondary prevention of myocardial infarction (MI) were encouraging[8]. In the Anturan Reinfarction Italian Study, patients with MI randomized to sulfinpyrazone (400 mg twice a day) were at lesser risk of suffering from reinfarction compared to the placebo group[8]. However, these results were disproved by a subsequent large trial in unstable angina[9] and the drug was never developed further for this indication.

Indobufene, 2–[*p*-(1-oxo-2-isoindolinyl)phenyl]butyric acid, is a competitive inhibitor of the cyclooxygenase moiety of the PGG/H synthase enzyme[10,11] and dose-dependently inhibits platelet aggregation in vitro and in vivo[12–15] and prolongs the bleeding time[16]. After a single oral dose of 200 mg indobufen, peak inhibition of TXB_2 production, using whole blood clotting, is at 2 h following administration[11]. After repeated administration, platelet inhibition lasts 12 hours after discontinuation of the drug[17]. Indobufen was shown to inhibit thrombosis in experimental animal models in vivo[18].

Indobufen has been investigated in clinical trials for preventing thromboembolism and graft closure. Indobufen, 100 mg b.i.d., was proven to reduce cardiovascular events, in particular stroke, in patients with heart disease associated with an increased risk of cardiogenic embolism[19]. The effect of indobufen on graft patency was investigated in patients with coronary artery disease and peripheral artery disease. In patients with coronary artery bypass grafting, indobufen at a daily dosage of 400 mg was as effective as aspirin plus dipyridamole in reducing graft closure[20]. Similar effects were observed in patients undergoing femoro-popliteal bypass[21]. In a pilot study, indobufen has been shown effective in the secondary prevention of major vascular events in non-rheumatic atrial fibrillation[22]. Further study is in progress to test the clinical efficacy of indobufen in this setting. Gastrointestinal drug-related adverse effects range from 3.9% to 23% in clinical trials[23].

Trifusal is a salicylic acid derivative that blocks COX-1 after transformation in vivo to the metabolite 2-hydroxy-4-trifluoromethyl-benzoate[24]. The use of trifusal in several clinical settings has given no convincing data for its use as an antiplatelet agent in human.

Serotonin antagonists

Serotonin (5-HT) acts on different cells by binding to specific receptors. Several 5-HT receptors have been identified and cloned, and the family of 5-HT receptors is the largest family of neurotransmitter receptors. Serotonin, accumulated in platelet dense granules by an active transport system, is liberated during the secretion reaction and acts as a positive feedback signal in the platelet activation process. The 5-HT_2 receptor, a G-protein coupled receptor, mediates amplification of platelet activation reactions induced by serotonin, and the prototype 5-HT_2 antagonist is ketanserin[25].

Ergoline derivatives LY53857, LY237733, sergolexole[26], and AT-1015[27] are as potent as ketanserin in inhibiting platelet aggregation. SR46349 and DV-7028 are highly potent 5-HT_2 receptor antagonists and exhibit antithrombotic action in animal models in vivo[28,29].

The effect of ketanserin (40 mg three times daily) on morbidity and mortality in patients with intermittent claudication was assessed by the PACK (Prevention of Atherosclerotic Complication with Ketanserin) study. Ketanserin was associated with an increased number of deaths in patients taking ketanserin and potassium-losing diuretics[30]. A secondary analysis, which excluded patients taking ketanserin and diuretics, showed a 23% reduction in major and minor cardiovascular events in patients taking ketanserin. Compared to placebo, ketanserin did not improve pain-free treadmill walking distance, but increased the ankle to arm systolic pressure ratio by decreasing the brachial systolic pressure[31].

Serious side effects, such as prolongation of QT and risk of torsade the pointes in patients receiving diuretics[32,33], potentially limit the use of ketanserin and other 5-HT_2 receptor blockers.

Thromboxane synthase inhibitors and TxA_2/PGH_2 receptor (TP) antagonists

For a long time, inhibition of thromboxane has been an attractive target for developing new antiplatelet drugs. TxA_2 synthesized in platelets by TxA_2 synthase, which uses PGH_2 as substrate, is a potent platelet activator and vasoconstrictor (Fig. 64.1). In addition, TxA_2 exerts additional vascular effects relevant to thrombosis and atherosclerosis, including proliferative effects[34] and stimulation of expression of adhesion molecules[35]. TxA_2 effects are mediated by a G protein-coupled receptor (TP). Thus, pharmacological blockade of TxA_2 can be achieved at two levels, the TxA_2 synthase and the TP (Fig. 64.1). Development of thromboxane synthase inhibitors was clinically relevant because these inhibitors could have overcome the unwanted effect of aspirin, i.e the broad suppression of eicosanoid synthesis both in platelets and endothelial cells. Dazoxiben is the prototype drug of TxA_2 synthase inhibitors[36], but the short half-life raised some concerns about the drug's capability to induce a constant suppression of TxA_2 synthesis in vivo[1]; the drug was evaluated in clinical trials with negative results and the development of the drug was abandoned. Other TxA_2 synthase inhibitors have been reported, including the imidazolyl derivatives Y-20811[37], CS-518[38] and OKY-046[39], and pyrazolotriazinyl alkanoic acids[40]. The development of specific TxA_2 synthase inhibitors was subsequently limited by the observation that both TxA_2 and its precursor PGH_2, which accumulates after TxA_2 synthase inhibition, act at the same receptor site. The blockade of TxA_2 synthase obstructs the arachidonic acid cascade with consequent accumulation of precursor substrates. Accumulated PGH_2 binds to TP as an agonist and potentially activates platelets (Fig. 65.1). In addition, the TP receptor is used by F_2-isoprostanes, potent platelet activators whose production is not affected by TxA_2 synthase inhibitors[41]. Experimental support to that concept has been given by in vitro and in vivo studies[42–44]. TxA_2 synthase inhibitors do not completely inhibit platelet aggregation, and have little effect on bleeding time and experimental thrombosis[42–44]. Dual inhibition of TxA_2 synthase and TP has resulted in a strong inhibition of platelet aggregation and prolongation of bleeding time, much greater than either drug alone[43]. In this context, the additional contribution to the impairment of hemostasis is given by PGI_2 formed by reorientation of the metabolism of the cyclic endoperoxides. Accumulated PGH_2 in platelets, as a result of TxA_2 synthase block, translocate to endothelial cells where they are transformed to PGI_2. Elegant demonstration of this concept was given by Gresele et al.[43], who showed that the effects of dual inhibition were reversed by blocking PGH synthase with aspirin. Thus, the development of new drugs has focused on dual inhibitors, enzyme plus receptor, and TP blockers (Fig. 64.1). The prototype drug of dual inhibitors is Ridogrel[45]. A series of new dual inhibitors is under investigation, including cinnaminophilin, a lignan isolated from *Cinnamonum philippinense*[46], and the non-carboxylic derivative BM-531[47]. The dual inhibitor picotamide, N,N′-bis[3-picolyl]-4-methoxy-isophthalamide, inhibits platelet aggregation induced by different agonists in vitro and ex vivo[48,49]. The drug is an inhibitor of thromboxane synthase[49], and competitive binding studies have shown that picotamide acts also as a TP antagonist[50].

Picotamide was tested on the clinical progression of

Cambridge Books Online © Cambridge University Press, 2009

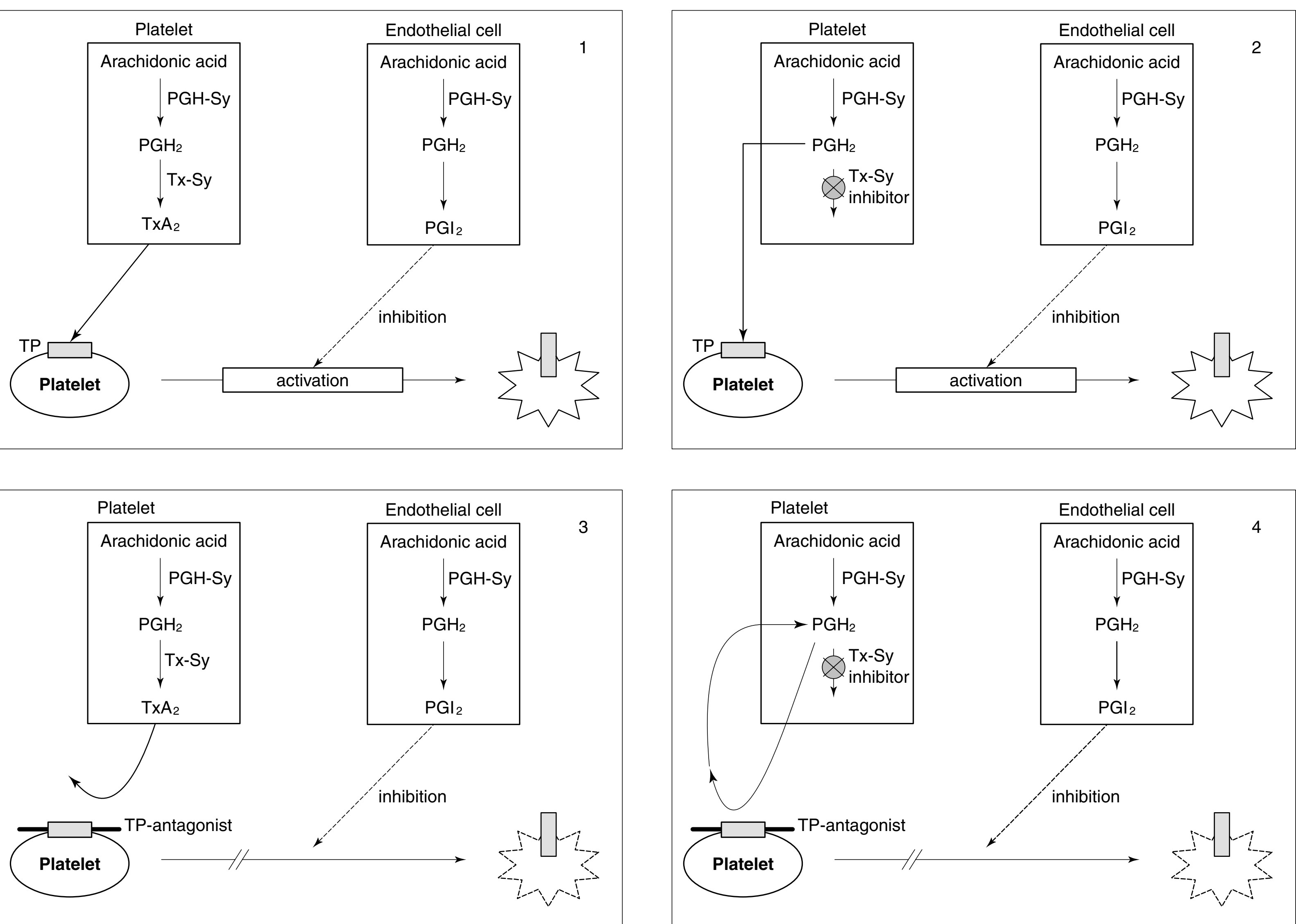


Fig. 64.1. Beyond aspirin-mediated inhibition of the arachidonic acid cascade: potential pharmacological interventions for blocking TxA_2-mediated platelet activation. Synthesis of TxA_2 is dependent on availability of free arachidonic acid, which is liberated from membrane phospholipids by phospholipases (not shown in the scheme), and on the activity of PGH_2- synthase (PG–Sy) and TxA_2-synthase (Tx–Sy); TxA_2 binds to a specific G protein-coupled receptor (TP) and initiates a series of intracellular events that leads to platelet activation (scheme 1). Specific TxA_2-synthase inhibitors block the production of TxA_2 with potential accumulation of its precursor PGH_2, which in turn may activate platelets by mimicking TxA_2 at the TP level (scheme 2). The combination of Tx–Sy inhibitors and TP inhibitors results in a marked inhibition of arachidonate-induced platelet aggregation and prolongation of bleeding time much greater than either drug alone (scheme 4). Contribution to this effect is given by metabolic reorientation of cyclic endoperoxide towards antiaggregatory prostaglandins, such as PGI_2 (scheme 4)[43].

Cambridge Books Online © Cambridge University Press, 2009

peripheral vascular disease in patients with intermittent claudication[51]. After 18 months follow-up there was no difference in terms of major and minor events in patients assigned to placebo or picotamide (intention to treat analysis)[51].

Several pharmacological TP inhibitors have been reported, including BM13.177, SQ-29,548, and GR32191[52–54], and the novel non-carboxylic derivatives BM-144 and BM-500 that are under investigation[55]. Two clinical trials were performed to test the effectiveness of TP inhibitors in reducing restenosis after PTCA. Both GR32191 and sulotroban, evaluated in the CARPORT (coronary artery restenosis prevention on repeated thromboxane A_2 antagonism) study[56] and in the M-HERT-II (multi-Hospital Eastern Atlantic restenosis trial) study, respectively[57], failed to prevent restenosis after PTCA. However, sulotroban was as effective as aspirin in preventing the occurrence of myocardial infarction[57].

Antithrombins and thrombin receptor antagonists

Thrombin is a serine protease that catalyses the cleavage of fibrinogen and of the platelet thrombin receptor. As a result thrombin activates both the coagulation pathway and platelets. As such, thrombin plays a central role in the mechanism of thrombosis. Antithrombins include the protease inhibitors r-hirudin and D-Phe–Pro–Arg–chloromethylketone that block the thrombin catalytic activity and are antithrombotic agents in vivo. These inhibitors are able to inactivate thrombus-bound thrombin and interrupt radiolabelled-platelet and radiolabelled-fibrinogen deposition in the vascular graft model[58]. In contrast, the heparin–ATIII complex is not able to affect thrombus formation in this model[58]. The clinical relevance of these findings has been supported by clinical trials with r-hirudin. In a randomized placebo-controlled study assessing subcutaneous hirudin vs. standard heparin or LMWH, hirudin reduced more effectively than heparin the rate of deep vein thrombosis and pulmonary embolism, without significant increase in bleeding[59]. Controlled clinical trials of bivalirudin[60–62] or hirudin[63,64] vs. standard heparin have also shown that antithrombins are effective in reducing reocclusion after thrombolysis, without producing significant bleeding. Randomized studies in patients undergoing PTCA have shown that hirudin or bivalirudin are comparable to heparin in protecting from angioplasty-related complications and are similar in frequency of bleeding[65–68]. In contrast, randomized placebo-controlled clinical trials in acute coronary syndromes have demonstrated that hirudin and bivalirudin do not cause significant clinical improvement compared to heparin, but increase intracranial bleeding[69–72].

This last evidence is in agreement with studies of experimental antithrombogenesis in baboons[58]. In this model the hirudin dose required to block platelet recruitment is at least one order of magnitude higher than the dose needed for inhibiting fibrin formation, and as a consequence a marked prolongation of clotting time, template bleeding, and surgical bleeding are observed[73]. Thus, some authors believe that antithrombin concentrations necessary to interrupt platelet activation in arterial thrombosis may never be achievable by systemic infusion without undesirable bleeding[73].

Thrombin receptors are G protein-coupled receptors activated by proteolytic cleavage (PAR receptors)[74]. Two main PAR receptors are specific for thrombin, PAR-1 and PAR-3[75]. PARs-targeted monoclonal antibodies are attractive candidates for receptor blockade. Strategies for developing antibodies against thrombin receptor are likely to require agents that inhibit both PAR-1 and PAR-3. Anti-PAR-1 monoclonal antibodies failed to fully prevent thrombin-induced platelet activation[76]. In contrast, anti-PAR-1 polyclonal antibodies, which probably cross-react with PAR-3, have provided protection from thrombosis in non human primates in vivo[77].

Cleavage of the receptor between Arg^{41} and Ser^{42} unmasks a new NH_2-terminal sequence that functions as a tethered ligand for the receptor. Peptides based on the new exposed sequence, from 5 (SFLLR) to 14 (SFLLRRNPNDKYEPF) amino acids, are potent thrombin receptor agonists[78]. Based on these sequences, receptor antagonist peptides have been constructed[79,80]. The undecapeptide C186–65 is a specific inhibitor of thrombin-mediated platelet activation[79]. Thus, thrombin receptor antagonists act differently from specific thrombin protease inhibitors and their effect is limited to platelet inhibition without affecting the clotting system. FR171113 is a selective non-peptide thrombin receptor antagonist, that is 50 times more potent than C186–65 in inhibiting thrombin-induced platelet aggregation (IC(50) 0.29 μM)[80]. FR171113 does not inhibit platelet aggregation induced by other agonists (ADP, collagen, arachidonic acid, U46619, PAF, adrenaline and calcium ionophore), and does not prolong the thrombin time, activated partial thromboplastin time, or prothrombin time[80].

Phosphodiesterase inhibitors

Cyclic AMP and GMP are intracellular second messengers that regulate physiological responses triggered by extracel-

Cambridge Books Online © Cambridge University Press, 2009

lular stimuli. Intracellular levels of cAMP and cGMP are regulated by the rate of synthesis and degradation operated by specific cyclases and phosphodiesterases (PDE), respectively. Most cells express one or more PDE isoenzymes that regulate intracellular cAMP or cGMP. At least nine different gene families of PDEs have been identified in mammalian tissues[81,82]. Platelets express PDE2, PDE3, and PDE5 isoenzymes. Specific inhibitors of PDEs inhibit platelet function in vitro and in vivo[83,84]. The antiplatelet drug cilostazol[85] is a selective inhibitor of PDE3 with IC(50) of 0.19 μM[86]. Cilostazol also has vasodilating activity due to inhibition of PDE3 in vascular smooth muscle cells, which also express PDE1, PDE4, and PDE5.

Cilostazol has been used for a decade in several countries for the treatment of peripheral arterial disease, and in 1999 was approved in the United States for intermittent claudication. In a recent study, 81 patients with intermittent claudication were randomly treated with cilostazol (200 mg/day for 12 weeks) or placebo. Patients receiving cilostazol showed a statistically significant increase in walking distance compared to placebo[87]. The efficacy of cilostazol combined with aspirin was also assessed in coronary stenting in comparison with ticlopidine plus aspirin[88]. The two treatment regimens were randomly assigned to 300 patients starting 2 days before stent implantation. Primary end point at 30 days was a composite of angiographic stent thrombosis and major cardiac events (death, myocardial infarction, bypass surgery, repeat intervention). The combination of cilostazol plus aspirin was as effective as aspirin plus ticlopidine.

Novel potent PDEs inhibitors have been synthesized. The cyclooctylurea derivative OPC-33540 inhibits recombinant PDE3 with IC^{50} of 0.32 nM[89]. The cyclohexenyl derivative NSP-513 is an orally active inhibitor of PDE3 with IC^{50} of 39 nM[90]. Aggregation of dog platelets induced by collagen and ADP is inhibited by NSP-513 with IC^{50} of 0.15 and 12 μM, respectively. NSP-513 administered to dogs inhibits ex vivo platelet aggregation and exhibits an antithrombotic activity in experimental femoral arterial thrombosis[90].

Dipyridamole (2,6-bis-diethanolamino-4,8-dipiperidino-pyrimido-(5,4-D)-pyrimidine), is an antithrombotic agent in experimental models and in several animal models acts as platelet inhibitor in vivo[91]. Dipyridamole has three main pharmacological activities relevant to platelet inhibition. First, dipyridamole is a potent inhibitor of phosphodiesterases[82,84], which control the intracellular levels of cAMP and cGMP, pivotal regulators of a wide variety of signal transduction pathways[83,84]. Secondly, dipyridamole is an inhibitor of adenosine reuptake by erythrocytes and endothelial cells[92,93]. Adenosine, which derives from ADP released during platelet aggregation, inhibits platelet function[94]. Thus, dipyridamole has been suggested to inhibit platelet function by locally increasing adenosine concentration during thrombus formation[94,95]. Third, dipyridamole is a potent chain breaking antioxidant[96,97]. Scavenging of free radicals, which inactivate the cyclooxygenase enzyme[98], could account for the delay in the exhaustion of platelet-inhibiting PGI_2[98–100]. In analogy with other antioxidants, dipyridamole could also affect free-radical mediated amplification of platelet activation[101].

Despite its pharmacological activities, the antiplatelet effect of dipyridamole is difficult to demonstrate in vitro. This is partly caused by a strong binding activity of dipyridamole to alpha-1-acid glycoprotein and consequent low free drug concentration in the platelet environment in vitro[102]. Thus, there is no clear-cut evidence of a specific platelet target for dipyridamole, and the mechanism of its antiplatelet activity is mainly speculative.

In the European Stroke Prevention Study 2 (ESPS-2), dipyridamole (200 mg twice a day) reduced the risk of stroke by 36.8% and the cumulative risk of stroke and death by 24.2% when compared with placebo[103]. Dipyridamole combined with 50 mg aspirin was also significantly more effective than either drug alone. This combination, approved by the FDA[104], is currently recommended as one of the first-line treatments for stroke prevention after a first transient ischemic attack or stroke[105].

Dipyridamole is FDA approved as an adjunct to warfarin for the prevention of postoperative thromboembolic complications of cardiac valve replacement with a mechanical prosthesis[106].

References

1 Reilly IA, FitzGerald GA. Inhibition of thromboxane formation in vivo and ex vivo: implications for therapy with platelet inhibitory drugs. *Blood* 1987; 69: 180–186.
2 Simon LS, Weaver AL, Graham DY et al. Anti-inflammatory and upper gastrointestinal effects of celecoxib in rheumatoid arthritis: a randomized controlled trial. *JAMA* 1999; 282: 1921–1928.
3 Agrawall NM, Van Kerckhove HEJM, Erhardt LJ, Geis GS. Misoprostol coadministered with diclofenac for prevention of gastroduodenal ulcers: a one-year study. *Dig Dis Sci* 1995; 40: 1125–1131.
4 Taha AS, Hudson N, Hawkey CJ et al. Famotidine for the prevention of gastric and duodenal ulcers caused by nonsteroidal antiinflammatory drugs. *N Engl J Med* 1996; 334: 1435–1439.
5 Cheatum DE, Arvanitakis C, Gumpel M, Stead H, Geis GS. An

Cambridge Books Online © Cambridge University Press, 2009

endoscopic study of gastroduodenal lesions induced by non-steroidal anti-inflammatory drugs. *Clin Ther* 1999; 21: 992–1003.

6 Pay GF, Wallis RB, Zelaschi D. The effect of sulfinpyrazone and its metabolites on platelet function in vitro and ex vivo. *Haemostasis* 1981; 10: 165–175.

7 Pedersen AK, FitzGerald GA. Cyclooxygenase inhibition, platelet function, and metabolite formation during chronic sulfinpyrazone dosing. *Clin Pharmacol Ther* 1985; 37: 36–42.

8 Sulfinpyrazone in post-myocardial infarction. Report from the Anturan Reinfarction Italian Study. *Lancet* 1982; 1: 237–242.

9 Cairns JA, Gent M, Singer J et al. Aspirin, sulfinpyrazone, or both in unstable angina. Results of a Canadian multicenter trial. *N Engl J Med.* 1985; 313: 1369–1375.

10 Patrignani P, Volpi D, Ferrario R, Romanzini L, Di Somma M, Patrono C. Effects of racemic, S- and R-indobufen on cyclooxygenase and lipoxygenase activities in human whole blood. *Eur J Pharmacol* 1990; 191: 83–88.

11 De Caterina R, Sicari R, Yan A et al. The d-enantiomer form of indobufen totally accounts for the anti-cyclooxygenase and antiplatelet activity ex vivo and for the increase in bleeding time by indobufen in man. *Thromb Haemost* 1992; 67: 258–263.

12 Bergamaschi M, Pierucci L, Branzoli U. Inhibitory activity of indobufen on platelet aggregation in vivo. *Pharmacol Res Commun* 1984; 16: 979–985.

13 Tremoli E, Maderna P, Sirtori M, Colli S, Corvi G, Sirtori CR. Indobufen treatment in type IIA hypercholesterolemic subjects: effects on platelet function and malondialdehyde production. *Pharmacol Res Commun* 1981; 13: 847–859.

14 Gursoy AZ, Yardimci TU, Emekli NB, Ugur MS, Ulutin ON. Preliminary studies on indobufen (K 3920). A new inhibitor of platelet aggregation. *Haematologica* 1981; 66: 756–767.

15 Cattaneo M, Bevilacqua C, Lecchi A, Mannucci PM. In vitro and ex vivo effects of indobufen on human platelet aggregation, the release reaction and thromboxane B2 production. *Haemostasis* 1987; 17: 293–300.

16 Vinazzer H, Fuccella LM. Clinical pharmacology studies with indobufen (K 3920): inhibitor of platelet aggregation. *J Clin Pharmacol* 1980; 20: 316–325.

17 Mannucci L, Maderna P, Colli S, Lavezzari M, Sirtori CR, Tremoli E. Indobufen is a potent inhibitor of whole blood aggregation in patients with a high atherosclerotic risk. *Thromb Res* 1987; 48: 417–426.

18 Di Minno G, Silver MJ. Mouse antithrombotic assay: a simple method for the evaluation of antithrombotic agents in vivo. Potentiation of antithrombotic activity of ethyl alcohol. *J Pharmacol Exp Ther* 1983; 225: 57–60.

19 Fornaro G, Rossi P, Mantica PG et al. Indobufen in the prevention of thromboembolic complications in patients with heart disease. A randomized, placebo-controlled, double-blind study. *Circulation* 1993; 87: 162–164.

20 Cataldo G, Heiman F, Lavezzari M, Marubini E. Indobufen compared with aspirin and dipyridamole on graft patency after coronary artery bypass surgery: results of a combined analysis. *Coron Artery Dis* 1998; 9: 217–222.

21 D'Addato M, Curti T, Bertini D et al. Indobufen vs. acetylsalicylic acid plus dipyridamole in long-term patency after femoropopliteal bypass. *Int Angiol* 1992; 11: 106–112.

22 Morocutti C, Amabile G, Fattaposta E et al. Indobufen versus warfarin in the secondary prevention of major vascular events in nonrheumatic atrial fibrillation. SIFA (Studio Italiano Fibrillazione Atriale) Investigators. *Stroke* 1997; 28: 1015–1021.

23 Wiseman LR, Fitton A, Buckley MN. Indobufen. A review of its pharmacodynamic and pharmakinetic properties, and therapeutic efficacy in cerebral, peripheral and coronary vascular disease. *Drugs* 1992; 44: 445–464.

24 De Castellarnau C, Sancho MJ, Vila L, Albors M, Rutllant ML. Effects and interaction studies of triflusal and other salicylic derivatives on cyclooxygenase in rats. *Prostaglandins Leukot Essent Fatty Acids* 1988; 31: 83–89.

25 Frishman WH, Huberfeld S, Okin S, Wang YH, Kumar A, Shareef B. Serotonin and serotonin antagonism in cardiovascular and non-cardiovascular disease. *J Clin Pharmacol* 1995; 35: 541–572.

26 McBride PA, Mann JJ, Nimchinsky E, Cohen ML. Inhibition of serotonin-amplified human platelet aggregation by ketanserin, ritanserin, and the ergoline 5HT2 receptor antagonists-LY53857, sergolexole, and LY237733. *Life Sci* 1990; 47: 2089–2095.

27 Kihara H, Hirose K, Koganei H et al. AT-1015, a novel serotonin (5-HT)2 receptor antagonist, blocks vascular and platelet 5-HT2A receptors and prevents the laurate-induced peripheral vascular lesion in rats. *J Cardiovasc Pharmacol* 2000; 35: 523–530.

28 Yang LH, Callas D, Herbert JM, Fareed J. Modulation of platelet function and vascular smooth muscle contractile actions by a novel, selective, highly potent 5-HT2 antagonist (SR46349). *Thromb Res* 1996; 81: 555–567.

29 Pawlak D, Pawlak K, Chabielska E et al. A potent 5-hydroxytryptmanine receptor (5-HT2A) antagonist, dv-7028 delays arterial thrombosis development in rats. *Thromb Res* 1998; 90: 259–270.

30 Prevention of Atherosclerotic Complications: controlled trial of ketanserin. Prevention of Atherosclerotic Complication with Ketanserin Trial Group. *BMJ* 1989; 298: 424–430.

31 Randomized placebo-controlled, double-blind trial of ketanserin in claudicants. Changes in claudication distance and ankle systolic pressure. PACK Claudication substudy. *Circulation* 1989; 80: 1544–1548.

32 Frishman WH, Huberfeld S, Okin S, Wang YH, Kumar A, Shareef B. Serotonin and serotonin antagonism in cardiovascular and non-cardiovascular disease. *J Clin Pharmacol* 1995; 35: 541–572.

33 Frishman WH, Grewall P. Serotonin and the heart. *Ann Med* 2000; 32: 195–209.

34 Cybulsky AV, Goodyer PR, Cyr MD, McTavish AJ. Eicosanoids enhance epidermal growth factor receptor activation and

Cambridge Books Online © Cambridge University Press, 2009

proliferation in glomerular epithelial cells. *Am J Physiol* 1992; 262: F639–F646.
35 Ishizuka T, Suzuki K, Kawakami M, Hidaka T, Matsuki Y, Nakamura H. Thromboxane A2 receptor blockade suppresses intercellular adhesion molecule-1 expression by stimulated vascular endothelial cells. *Eur J Pharmacol* 1996; 312: 367–377.
36 Vermylen J, Defreyn G, Carreras LO, Machin SJ, Van Schaeren J, Verstraete M. Thromboxane synthetase inhibition as antithrombotic strategy. *Lancet* 1981; 1: 1073–1075.
37 Mikashima H, Ochi H, Muramoto Y, Hirotsu K, Arima N. Irreversible inhibition of thromboxane (TX) A2 synthesis by Y-20811, a selective TX synthetase inhibitor. *Biochem Pharmacol* 1992; 43: 295–299.
38 Asai F, Ito T, Ushiyama S, Matsuda K, Oshima T. In vitro antiplatelet profiles of the new thromboxane synthetase inhibitor sodium 2–(1-imidazolylmethyl)-4,5-dihydrobenzo[b]thiophene-6-carboxylate. *Arzneimittelforschung* 1991; 41: 506–510.
39 Ichikawa K, Tazawa S, Hamano S, Kojima M, Hiraku S.Effect of ozagrel on locomotor and motor coordination after transient cerebral ischemia in experimental animal models. *Pharmacology* 1999; 59: 257–265.
40 Lasserre B, Mavel S, Coudert P et al. Thromboxane A2 biosynthesis inhibitors: synthesis and evaluation of pyrazolotriazinyl alkanoic acids. *Prostaglandins Leukot Essent Fatty Acids* 1994; 51: 157–161.
41 Audoly LP, Rocca B, Fabre JE et al. Cardiovascular responses to the isoprostanes iPF(2alpha)-III and iPE(2)-III are mediated via the thromboxane A(2) receptor in vivo. *Circulation* 2000; 101: 2833–2840.
42 FitzGerald GA, Reilly IAG, Pedersen AK. The biochemical pharmacology of thromboxane synthase inhibition in man. *Circulation* 1985; 72: 1194–1201.
43 Gresele P, Arnout J, Deckmyn H, Huybreckts E, Pieters G, Vermylen J. Role of proaggretatory and antiaggregatory prostaglandins in hemostasis. Study with combined thromboxane synthase inhibition and thromboxane receptor antagonism. *J Clin Invest* 1987; 80: 1435–1445.
44 FitzGerald DJ, Fragetta J, FitzGerald GA. Prostaglandin endoperoxides modulate the response to thromboxane synthase inhibition during coronary thrombosis. *J Clin Invest* 1988; 82: 1708–1713.
45 Heylen L, De Clerck F, Somers Y, Leysen JE. Thromboxane A2/prostaglandin endoperoxide (TXA2/PG-END) receptor binding properties in human platelets of ridogrel, a combined TXA2 synthase inhibitor – TXA2/PG-END receptor antagonist. *Blood Coagul Fibrinol* 1991; 2: 617–621.
46 Yu SM, Wu TS, Teng CM. Pharmacological characterization of cinnamophilin, a novel dual inhibitor of thromboxane synthase and thromboxane A2 receptor. *Br J Pharmacol* 1994; 111: 906–912.
47 Dogne JM, de Leval X, Neven P et al. Effects of a novel noncarboxylic thromboxane A2 receptor antagonist (BM-531) derived from torasemide on platelet function. *Prostaglandins Leukot Essent Fatty Acids* 2000; 62: 311–317.
48 Violi F, Ghiselli A, Iuliano L, Pratico D, Alessandri C, Balsano F. Inhibition by picotamide of thromboxane production in vitro and ex vivo. *Eur J Clin Pharmacol* 1988; 33: 599–602.
49 Gresele P, Deckmyn H, Arnout J, Nenci GG, Vermylen J. Characterization of *N,N′*-bis(3-picolyl)-4-methoxy-isophthalamide (picotamide) as a dual thromboxane synthase inhibitor/thromboxane A2 receptor antagonist in human platelets. *Thromb Haemost* 1989; 61: 479–484.
50 Modesti PA, Cecioni I, Colella A, Costoli A, Paniccia R, Neri Serneri GG. Binding kinetics and antiplatelet activities of picotamide, a thromboxane A2 receptor antagonist. *Br J Pharmacol* 1994; 112: 81–86.
51 Balsano F, Violi F. Effect of picotamide on the clinical progression of peripheral vascular disease. A double-blind placebo-controlled study. The ADEP Group. *Circulation* 1993; 87: 1563–1569.
52 Gresele P, Arnout J, Janssens W, Deckmyn H, Lemmens J, Vermylen J. BM13.177, a selective blocker of platelet and vessel wall thromboxane receptors is active in man. *Lancet* 1984; i: 991–994.
53 Darius H, Smith JB, Lefer AM. Beneficial effects of a new potent and specific thromboxane receptor antagonist (SQ-29,548) in vitro and in vivo. *J Pharmacol Exp Ther* 1985; 253: 274–281.
54 Humphrey PPA, Hallet P, Horby EJ, Wollis CJ, Collington EW, Lumley P. Pathophysiological actions of thromboxane A2 and their pharmacological antagonism by thromboxane receptor blockade with GR32191. *Circulation* 1990; 81 (suppl I): I42–I52.
55 Masereel B, Damas J, Fontaine J et al. Thromboxane A2 receptor antagonism in man and rat by a sulphonylcyanoguanidine (BM-144) and a sulphonylurea (BM-500). *J Pharm Pharmacol* 1999; 51: 695–701.
56 Serruys PW, Rutsch W, Heyndrickx GR et al. Prevention of restenosis after percutaneous transluminal coronary angioplasty with thromboxane A2 receptor blockade. A randomized, double-blind, placebo controlled trial. Coronary Artery Restenosis Prevention on Repeated Thromboxane A_2 Antagonism Study (CARPORT). *Circulation* 1991; 84: 1568–1580.
57 Savage MP, Goldberg S, Bove AA et al. Effect of thromboxane A2 blockade on clinical outcome and restenosis after successful coronary angioplasty. Multi Hospital Eastern Atlantic Restenosis Trial (M-HERT-II). *Circulation* 1995; 92: 3194–3200.
58 Kelly AB, Marzec UM, Krupski W, Bass A, Cadroy Y, Hanson SR, Harker LA. Hirudin interruption of heparin-resistant arterial thrombus formation in baboons. *Blood* 1991; 77: 1006–1012.
59 Eriksson BI, Ekman S, Kalebo P, Zachrisson B, Bach D, Close P. Prevention of deep-vein thrombosis after total hip replacement: direct thrombin inhibition with recombinant hirudin, CGP 39393. *Lancet* 1996; 347: 635–639.
60 Lidon RM, Theroux P, Lesperance J et al. A pilot, early angiographic patency study using a direct thrombin inhibitor as

Cambridge Books Online © Cambridge University Press, 2009

adjunctive therapy to streptokinase in acute myocardial infarction. *Circulation* 1994; 89: 1567–1572.

61 Theroux P, Perez-Villa F, Waters D, Lesperance J, Shabani F, Bonan R. Randomized double-blind comparison of two doses of Hirulog with heparin as adjunctive therapy to streptokinase to promote early patency of the infarct-related artery in acute myocardial infarction. *Circulation* 1995; 91: 2132–2139.

62 White HD, Aylward PE, Frey MJ et al. Randomized, double-blind comparison of hirulog versus heparin in patients receiving streptokinase and aspirin for acute myocardial infarction (HERO). Hirulog Early Reperfusion/Occlusion (HERO) Trial Investigators. *Circulation* 1997; 96: 2155–2161.

63 Antman EM. Hirudin in acute myocardial infarction. Thrombolysis and Thrombin Inhibition in Myocardial Infarction (TIMI) 9B trial. *Circulation* 1996; 94: 911–921.

64 Antman EM. Hirudin in acute myocardial infarction. Safety report from the Thrombolysis and Thrombin Inhibition in Myocardial Infarction (TIMI) 9A Trial. *Circulation* 1994; 90: 1624–1630.

65 Topol EJ, Bonan R, Jewitt D et al. Use of a direct antithrombin, hirulog, in place of heparin during coronary angioplasty. *Circulation* 1993; 87: 1622–1629.

66 Bittl JA. Comparative safety profiles of hirulog and heparin in patients undergoing coronary angioplasty. The Hirulog Angioplasty Study Investigators. *Am Heart J* 1995; 130: 658–665.

67 van den Bos AA, Deckers JW, Heyndrickx GR et al. Safety and efficacy of recombinant hirudin (CGP 39 393) versus heparin in patients with stable angina undergoing coronary angioplasty. *Circulation* 1993; 88: 2058–2066.

68 Zoldhelyi P, Webster MW, Fuster V et al. Recombinant hirudin in patients with chronic, stable coronary artery disease. Safety, half-life, and effect on coagulation parameters. *Circulation* 1993; 88: 2015–2022.

69 GUSTOIIa investigators. Randomized trial of intravenous heparin versus recombinant hirudin for acute coronary syndromes. *Circulation* 1994; 60: 1631–1637.

70 Cannon CP, Maraganore JM, Loscalzo J et al. Anticoagulant effects of hirulog, a novel thrombin inhibitor, in patients with coronary artery disease. *Am J Cardiol* 1993; 71: 778–782.

71 Bittl JA, Strony J, Brinker JA et al. Treatment with bivalirudin (Hirulog) as compared with heparin during coronary angioplasty for unstable or postinfarction angina. Hirulog Angioplasty Study Investigators. *N Engl J Med* 1995; 333: 764–769.

72 Fuchs J, Cannon CP. Hirulog in the treatment of unstable angina. Results of the Thrombin Inhibition in Myocardial Ischemia (TIMI) 7 trial. *Circulation* 1995; 92: 727–733.

73 Harker LA, Hanson SR, Kelly AB. Antithrombotic strategies targeting thrombin activities, thrombin receptors and thrombin generation. *Thromb Haemost* 1997; 78: 736–741.

74 Coughlin SR, Vu TK, Hung DT, Wheaton VI. Characterization of a functional thrombin receptor. Issues and opportunities. *J Clin Invest* 1992; 89: 351–355.

75 Ishihara H, Connolly AJ, Zeng D et al. Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature* 1997; 386: 502–506.

76 Klement P, Borm A, Hirsh J, Maragonore J, Wilson G, Weitz J. The effect of thrombin inhibitors on tissue plasminogen activator induced thrombolysis in rat model. *Thromb Haemost* 1992; 68: 64–68.

77 Cook JJ, Sitko GR, Bednar B et al. An antibody against the exosite of the cloned thrombin receptor inhibits experimental arterial thrombosis in the African green monkey. *Circulation* 1995; 91: 2961–2971.

78 Hui KY, Jakubowsky JA, Wyss VL, Angleton EL. Minimal sequence on thrombin receptor agonist peptide. *Biochem Biophys Res Commun* 1992; 184: 790–796.

79 Scarborough RM. C186–65, a thrombin receptor antagonist designed from tethered ligand agonist peptides. *Circulation* 1992; 86 (Suppl. I): 1–151.

80 Kato Y, Kita Y, Nishio M et al. In vitro antiplatelet profile of FR171113, a novel non-peptide thrombin receptor antagonist. *Eur J Pharmacol* 1999; 384: 197–202.

81 Beavo JA, Conti M, Heaslip RJ. Multiple cyclic nucleotide phosphodiesterases. *Mol Pharmacol* 1994; 46: 399–405.

82 Soderling SH, Bayuga SJ, Beavo JA. Identification and characterization of a novel family of cyclic nucleotide phosphodiesterase. *J Biol Chem* 1998; 273: 15553–15558.

83 Mills DCB, Smith JB. The influence on platelet aggregation of drugs that affect the accumulation of adenosine 3′-5′-cyclic monophosphate in platelets. *Biochem J* 1971; 121: 185–196.

84 Fawcett L, Baxendale R, Stacey P et al. Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A. *Proc Natl Acad Sci* 2000; 97: 3702–3707.

85 Kimura Y, Tani T, Kanbe T, Watanabe K. Effect of cilostazol on platelet aggregation and experimental thrombosis. *Arzneimitelforschung* 1985; 35: 1144–1149.

86 Umekawa H, Tanaka T, Kimura Y, Hidaka H. Purification of cyclic adenosine monophosphate phosphodiesterase from human platelets using new-inhibitor Sepharose chromatography. *Biochem Pharmacol* 1984; 33: 3339–3344.

87 Dawson DL, Cutler BS, Meissner MH, Strandness DE Jr. Cilostazol has beneficial effects in treatment of intermittent claudication: results from a multicenter, randomized, prospective, double-blind trial. *Circulation* 1998; 98: 678–686.

88 Yoon Y, Shim WH, Lee DH et al. Usefulness of cilostazol versus ticlopidine in coronary artery stenting. *Am J Cardiol* 1999; 84: 1375–1380.

89 Sudo T, Tachibana K, Toga K et al. Potent effects of novel antiplatelet aggregatory cilostamide analogues on recombinant cyclic nucleotide phosphodiesterase isozyme activity. *Biochem Pharmacol* 2000; 59: 347–356.

90 Hirose H, Mashiko S, Kimura T et al. Antithrombotic activity of NSP-513, a novel selective phosphodiesterase 3 inhibitor, on femoral arterial thrombosis induced by physical stenosis and electrical current: comparison of antithrombotic and hemodynamic effects. *J Cardiovasc Pharmacol* 2000; 35: 586–594.

Cambridge Books Online © Cambridge University Press, 2009

91 FitzGerald GA. Dipyridamole. *N Engl J Med* 1987; 316: 1247–1257.
92 Schrader J, Berne RM, Rubio R. Uptake and metabolism of adenosine by human erythrocyte ghosts. *Am J Physiol* 1972; 223: 159–166.
93 Crutchley DJ, Ryan US, Ryan JW. Effects of aspirin and dipyridamole on the degradation of adenosine diphosphate by cultured cells derived from bovine pulmonary artery. *J Clin Invest* 1980; 66: 29–35.
94 Born GVR, Mils DCB. Potentiation of the inhibitory effect of adenosine on platelet aggregation by drugs that prevent its uptake. *Proc Physiol Soc* 1969; 41P–42P.
95 Haslam RJ, Lynham JA. Activation and inhibition of blood platelet adenylate-cyclase by adenosine or by 2-chloroadenosine. *Life Sci* 1972; 11 (part2): 1143–1154.
96 Iuliano L, Pedersen JZ, Rotilio G, Ferro D, Violi F. A potent chain-breaking antioxidant activity of the cardiovascular drug dipyridamole. *Free Radic Biol Med* 1995; 18: 239–247.
97 Iuliano L, Piccheri C, Coppola I, Pratico D, Micheletta F, Violi F. Fluorescence quenching of dipyridamole associated to peroxyl radical scavenging: a versatile probe to measure the chain breaking antioxidant activity of biomolecules. *Biochim Biophys Acta* 2000; 1474: 177–182.
98 Marnett LJ, Siedlik PH, Ochs RC et al. Mechanism of the stimulation of prostaglandin H synthase and prostacyclin synthase by the antithrombotic and antimetastatic agent, nafazatrom. *Mol Pharmacol* 1984; 26: 328–335.
99 Deckmyn H, Gresele P, Arnout J, Todisco A, Vermylen J. Prolonging prostacyclin production by nafazatrom or dipyridamole. *Lancet* 1984; ii: 410–411.
100 Boeynaems JM, Van Coevorden A, Demolle D. Dipyridamole and vascular prostacyclin production. *Biochem Pharmacol* 1986; 35: 2897–2902.
101 Iuliano L, Colavita AR, Leo R, Pratico D, Violi F. Oxygen free radicals and platelet activation. *Free Radic Biol Med* 1997; 22: 999–1006.
102 El-Gamel S, Wollert U, Muller WE. Optical studies on the specific interaction of dipyridamole with alpha 1-acid glycoprotein (orsomucoid). *J Pharm Pharmacol* 1982; 34: 152–157.
103 Diener HC, Cunha L, Forbes C, Sivenius J, Smets P, Lowenthal A. European Stroke prevention study-2. Dipyridamole and acetylsalicylic acid in the secondary prevention of stroke. *J Neurol Sci* 1996; 143: 1–13.
104 SoRelle R. FDA approves new drug to reduce risk of stroke. *Circulation* 2000; 101: e41.
105 Sacco RL, Elkind MS. Update on antiplatelet therapy for stroke prevention. *Arch Intern Med* 2000; 160: 1579–1582.
106 Pouler H, Buyse M. Effects of dipyridamole in combination with anticoagulant therapy on survival and thromboembolic events in patients with prosthetic heart valves. A meta-analysis of the randomized trials. *J Thorac Cardiovasc Surg* 1995; 110: 463–472.

Cambridge Books Online © Cambridge University Press, 2009

65

Pharmacogenetics as a new antiplatelet strategy

Giovanni de Gaetano*, Chiara Cerletti and Licia Iacoviello

Department of Vascular Medicine and Pharmacology, Istituto di Ricerche Farmacologiche Mario Negri, Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy and Centro di Ricerca e Formazione ad Alta Tecnologia nelle Scienze Biomediche, Università Cattolica, Campobasso, Italy

"At that time two men will be working in a field:
one will be taken away, the other will be left behind.
Two women will be at a mill grinding meal:
one will be taken away, the other will be left behind."
Mt. 24, 40–41

Antiplatelet strategy: a brief historical review

The first three drugs tested in clinical trials for their potential antiplatelet effects were all already used in current practice for different reasons, unrelated to thrombosis and ischemic artery disease[1].

Aspirin was known as an anti-inflammatory compound, sulfinpyrazone was used for its uricosuric properties and dipyridamole was endowed with vasodilatory (coronarodilatory) effect. Their mechanism of action, as antiplatelet agents, was largely unknown, but the possibility of replacing oral anticoagulants with relatively safer substances, not requiring any laboratory control, convinced many clinicians to adapt an antiplatelet strategy, even before the results of well-planned and conducted clinical trials were made available.

The discovery of the crucial role of prostaglandins and thromboxane A2 (TxA2) in platelet function and of their possibly unselective pharmacological control by aspirin, stimulated for the first time the search for a more rational antiplatelet strategy.

However, the development of selective TxA2-synthase inhibitors, TxA2-receptor antagonists and of compounds endowed with both properties was not followed by the expected clinical results. This was due to several reasons, including the complex re-orientation of platelet arachidonate metabolism following pharmacological suppression of TxA2 synthesis or of its receptor function[2–4].

In the meantime, ticlopidine, a new compound, whose mechanism of action was unrelated to arachidonate metabolism was made available: this drug inhibited platelet function, particularly ADP-induced platelet aggregation[1]. Some workers suggested a parallel between the effect of ticlopidine and the congenital platelet defect observed in a rare hemorrhagic disorder, Glanzmann's thrombasthenia[5]. The assumption was incomplete, but had the great merit to orient new antiplatelet strategy towards mimicking naturally occurring platelet disorders. The role of ADP as the princeps of platelet function agonists was discovered in 1960, but the molecular basis of its effects is just beginning to be understood. A model of three purinergic receptors, each contributing separately to ADP-induced mechanisms has recently been proposed[6].

Following the results of clinical trials suggesting that ticlopidine, an inhibitor of ADP-induced platelet aggregation, might be at least as effective as aspirin, the search for other anti-ADP selective agents led to the development of clopidogrel[7]. The antiaggregating activity of this compound has been attributed to an active metabolite, which irreversibly affects the ADP receptor P2Y12[8]. More recently selective, specific and competitive P2Y12 antagonists have been identified. Experience gained with intravenous administration of these compounds has encouraged the development of orally active P2Y12 receptor antagonists with the expectation that these compounds will have significant advantages over other existing or emerging oral antiplatelet agents[9].

The concomitant progress in biochemical and molecular mechanisms regulating platelet function made it possible to develop RGD peptides, and to identify some snake venoms containing RGD proteins. RGD is a triplet of amino acids (Arg–Gly–Asp) which allow fibrinogen binding to the

platelet glycoprotein GPIIb–IIIa. The latter is lacking or dysfunctional in Glanzmann's thrombasthenia[10].

Another obvious approach to control platelet function was to develop monoclonal antibodies against the GPIIb–IIIa (the first humanized antibody, currently used with success in some clinical conditions was abciximab or c7E3 or ReoPro)[11]. More recently, GPIIb–IIIa antagonists have been developed, based on the RGD sequence (or on a KGD sequence where Arg is substituted by Lys). Orally active inhibitors of the GPIIb–IIIa receptor have been developed as well. They are inherently active or are metabolized to the active form after ingestion[12]. The hypothesis that blockade of the GPIIb–IIIa function would be translated into a reduction in short-term ischemic complications and improvement in long-term clinical outcomes has only been partially met. The recent publication of the results of clinical trials with different oral GPIIb–IIIa inhibitory drugs has generated a serious disappointment on the true clinical benefit of this antiplatelet strategy[13].

From a conceptual point of view an antiplatelet strategy similar to that mentioned above can be considered in relation to the glycoprotein GPIb and its major binding protein, namely von Willebrand factor[14].

In conclusion, since the 1970s a great effort was made to improve the efficacy of the antiplatelet strategy by developing new drugs based on the discovery of new biochemical mechanisms underlying platelet function. Such an effort has been paralleled by the performance of dozens of clinical trials on old and new antiplatelet drugs. Both individual trials[7,15–17] and accurate metanalysis of hundreds of trials of different size[18] have provided fundamental indications for therapy and prevention of major vascular events such as myocardial infarction, stroke and vascular mortality.

Populations rather than individuals as traditional targets of antiplatelet strategy

The indications obtained by traditional trials are well adapted to wide populations, but not as well to individual patients. The transferability of clinical data to the greatest number of patients is an important result of clinical trials such as ISIS-2[15]: one of the most useful advancements in the cardiovascular therapy during the past decades has been the demonstration of the efficacy to take an aspirin as soon as possible when myocardial infarction has only been suspected on the basis of a clinical judgement, without waiting for laboratory enzyme measurements or ECG confirmation. However, clinicians would like to have clearer indications, if not for any single patient, at least for less heterogeneous groups than those identified by epidemiologists.

Table 65.1. Results of a current hypothetical trial with an antiplatelet drug

Patients	Control treatment	Antiplatelet drug
Treated	100	100
No event	90	93
With events	10	7
Benefit from the drug	0	3

Note:
The beneficial effect of the antiplatelet drug is 30%, but only three out of 100 treated patients will be 'saved' by the treatment. At the moment, there is little chance to identify *a priori* the 90 patients who will have no benefit from the drug, the ten patients who will suffer an event despite the treatment and the three patients who will gain the maximum benefit from the drug (based on ref.[19]).

If, in a simplified hypothetical trial, one treats for 1 year 100 heart attack patients with the best available therapy plus an antiplatelet drug, seven out of the ten patients who would have died without that drug will die anyway, three will be saved by the antiplatelet treatment and the other 90 will only be exposed to the risks of treatment without having any benefits. The problem is that we are presently unable to identify *a priori* the three patients who would gain their maximum benefits from an antiplatelet drug or even the patients who most likely will suffer a new vascular event or will die[19] (Table 65.1).

In the last few years, some possibilities have been proposed to select *a priori* groups of subjects who could benefit from aspirin or other antiplatelet therapy, with results which could be more targeted to them.

An observation derived from the Physicians' Health Study[20] has shown that individuals who had lower C reactive protein, a marker of inflammation and a prognostic parameter in severe unstable angina[21], had little or no protection from aspirin against cardiovascular events, whereas aspirin was particularly active in those with higher levels of this protein[22].

The significant association between C reactive protein and the clinical effectiveness of aspirin might be related to reduction by this drug of the inflammatory components that might play a pathogenetic role in acute coronary syndromes[23]. Although this interpretation can be challenged on the basis of the relatively low, apparently non-anti-inflammatory dose of aspirin used in the Physicians' Health Study, the possibility to include in future primary prevention studies only those subjects with high levels of C

Cambridge Books Online © Cambridge University Press, 2009

reactive protein (or some other easily measurable parameter) is attracting attention. One can argue that C reactive protein does not directly distinguish 'responders' and 'non-responders' to aspirin, but identifies higher risk subjects for whom it will be easier to highlight the evidence of aspirin efficacy[19].

This concept is in apparent contrast with the conclusions of a recent study[24] suggesting that the lower the blood pressure, the greater the effectiveness of aspirin. Moreover, subjects with higher blood pressure (systolic values above 145 mmHg) not only did not benefit from aspirin but were exposed to a serious risk of hemorrhage. Thus, whatever the relationship between aspirin effect and laboratory (C reactive protein) or clinical (hypertension) parameters, it is ethical to reduce the number of subjects and patients exposed to the side effects of aspirin (or other antiplatelet drug) without any clinical benefits.

The use of aspirin and other antiplatelet drugs prescribed today or presently in the development phase is thus limited by suboptimal efficacy that results largely from our inability to account for interindividual differences in disease etiology and drug response[19,23].

The development of pharmacogenetics and individualized medicine

These interindividual differences are determined, to a large extent, by inherited predispositions and susceptibilities. Knowledge of the genetic differences that explain these individual characteristics, and, based upon it, the development of specific diagnostics and therapeutics, will therefore be critical for the successful transition to a future progress in health care.

Today, a typical drug development programme usually proceeds on the broad assumption that subjects (or patients) are homogeneous groups with little interindividual variability. However, the efficacy of a drug may display differences between patients, some of whom may suffer adverse drug reactions that do not occur in others taking the same drug at the same dose.

It is the impact of these individual genetic differences on the risk/benefit ratio and on improved therapeutic use of drugs that the science of pharmacogenetics aims to unravel. This subject has been recently reviewed by several authors[25–38].

While mutation is a rare variation in DNA sequence, which is found in less than 1% of the population, polymorphism indicates a difference in DNA sequence among individuals that occurs in 1% or more of the population. Sometimes up to 50% of a population is carrying a given polymorphism. Each individual is characterized by a sequence of three billion base pairs. One out of 300 base pairs varies from person to person, which means that any individual can be distinguished from his neighbour by about 10 million base pairs. As only 1% of the base pairs appears to be functional, about 100 000 base pairs account for changes in protein structure and function. This common variation is called polymorphism and single nucleotide polymorphism (SNP) is the most common form, where the DNA varies by only a single base or nucleotide. Identification and mapping of SNP will provide new markers across the genome that will help identify regions of the genome itself associated with therapeutic response or adverse effects. Presently, pharmacogenetic studies are mainly based on correlating individual response with variations in 'candidate' genes involved in the drug's mode of action or metabolic pathways[38].

Pharmacogenetics may be defined as the variability in drug response due to genetic factors. Response is used here in the broadest sense: absorption, distribution, metabolism, excretion pathways, target receptors, drug's mechanism of action, efficacy, side effects.

From an experimental point of view, having a certain result in a pharmacogenetic test would become part of the inclusion criteria for enrolling patients in clinical trials.

This new scientific approach would have the potential to enable physicians to identify susceptible patients and enhance the risk/benefit ratio for a given drug. One could envision a day when physicians will use pharmacogenetic rather than population data to make more informed decisions about therapy, especially in those multifactorial conditions such as cardiovascular disease.

It is important, however, to have realistic expectations about pharmacogenetics, at least in the next few years. The analysis of a broad set of genetic markers may only show that a genotypically defined subgroup of patients with a certain disease may have a higher probability of responding in a certain way to a certain drug. In this respect pharmacogenetic analysis has been compared to the assessment of blood pressure or cholesterol levels: when raised, these parameters are accepted risk factors for cardiovascular disease, but they do not necessarily imply that the patient will definitely suffer from a specific cardiovascular disease.

Moreover, cardiovascular disease, like other chronic diseases, is multifactorial and is caused by a combination of several genes and environment or non-genetic factors such as diet and physical excercise. These diseases are therefore difficult to dissect and the degree of correlation between genotypes and disease or drug response may be quite low. These days, genetic epidemiology is focusing

Cambridge Books Online © Cambridge University Press, 2009

increasing effort on controlling for potential environmental confounders.

Adverse events associated with drugs remain a significant problem[32]. Despite the large numbers typically enrolled in clinical trials to evaluate efficacy and safety of new drugs, rare adverse reactions (less than 1 in 1000) tend to be identified only when the drug has been used in much broader patient populations, i.e. during the initial marketing phase.

Pharmacogenetics could overcome this difficulty by targeting drugs to patients likely to benefit and unlikely to experience adverse events.

If a new molecule is developed for the treatment of acute myocardial infarction and, in phase II trials definite measures of efficacy are demonstrated in only 30% of patients, a phase III placebo-controlled trial would need to be very large, because 70% of patients would be predicted not to respond to the drug[34]. As no one would know which 70% would be 'non-responders', these patients would, in effect, participate in the trial solely for the purpose of revealing adverse reactions. Using a SNP profile one could identify in the phase II trial, the 30% of patients likely to respond. A phase III trial could then recruit only those patients with that pharmacogenetic profile, and trials could be smaller, faster and less expensive. Inclusion of patients whose pharmacogenetic profiles indicate a low chance of efficacy would expose them to potential adverse reactions and would be ethically questionable.

A 'single nucleotide polymorphisms' (SNP) Consortium has been recently formed to advance the development of genetic-based diagnostics and therapeutics, through the creation of a high quality, high density SNP map of the human genome, which will be made available to all parties at no cost. The SNP Consortium is a public initiative funded by the Wellcome Trust and 11 major pharmaceutical companies and contracts SNP research to leading academic centres. The creation of this genome-wide map of SNP, together with the recent completion of the human genome sequence, is expected to greatly expedite genetic research in the next few years[38].

Finally, race or ethnic specificity must be considered in relation to the population frequency of polymorphic genes of pharmacogenetic interest, as recently reviewed by Weber[39]. Association of these genes with person-to-person differences in drug effectiveness and toxicity may also depend on the racial or ethnic characteristics of a population. Information about ethnic specificity is an integral part of pharmacogenetics. For example, primaquine sensitivity, a sex-linked trait attributed to glucose-6-phosphate dehydrogenase deficiency, mainly affects males among African, Mediterranean and Oriental people. Other examples include the remarkable sensitivity of Japanese to alcohol (ethanol) compared with whites and the ethnic specificity of the cytochrome P-450 enzyme CYP2D6* polymorphism, that results in poor, extensive and ultrarapid metabolizers of at least 30 drugs, including warfarin. CYP2C19* polymorphism, which accounts for variable metabolism of proguanyl, omeprazole and certain barbiturates, is present in 3–6% of whites, but in 8–23% of Asians. The polymorphic (*N*-acyl-transferase2, NAT2*) acetylation of hydrazine and aromatic amine drugs, such as isoniazid, has been found in 40–70% of whites, in 50–60% of black Africans, but only in 10–20% of Asians[39,32].

Some examples of possible variability in antiplatelet and other antithrombotic drug response due to genetic factors

Data is still lacking on the genetic control of antiplatelet drugs' pharmacokinetics, but an instructive example of the importance of pharmacogenetics in drug metabolism and pharmacological response is given by a common and quite old antithrombotic compound such as warfarin.

Polymorphisms and the efficacy and safety of the oral anticoagulant warfarin

Warfarin is the oral anticoagulant drug most widely used in patients with venous and arterial thromboembolism. To keep the WHO international normalized ratio (INR) values between 2.0 and 3.0, doses of warfarin may widely fluctuate, from 1 to 20 mg or more daily. Standardized induction schemes with monitoring of INR over the first 4 days have only a 69% success rate. Such interindividual variations are partly due to the presence of polymorphism of the CYP2C9 gene[40]. CYP2C9 is a cytochrome P450 enzyme responsible for the metabolism of S-warfarin. S-warfarin, the more potent isoform of warfarin, is converted by CYP2C9 into inactive 6-hydroxy and 7-hydroxy metabolites.

CYP2C9*2 and CYP2C9*3 polymorphisms are both point mutations of the wild-type gene CYP2C9. In vitro data show that the CYP2C9*3 variant is less than 5% and the CYP2C9*2 about 12% as efficient as the wild-type enzyme. In vivo data show that the presence of the mutant polymorphism is responsible for impaired metabolism of warfarin, as carriers of either mutant allelic form behave as 'poor' warfarin metabolizers. Up to 20% of the general population could be carrier of at least one of these variations.

To verify the impact of the CYP2C9 gene polymorphism on the warfarin dose requirements, a study has been

Cambridge Books Online © Cambridge University Press, 2009

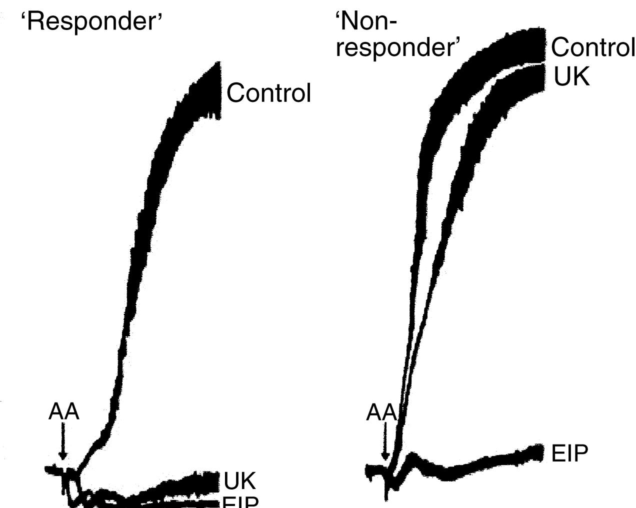


Fig. 65.1. Effect of dazoxiben (UK), a thromboxane A2-synthase inhibitor, and of 9,11-epoxyiminoprosta-5,13-dienoic acid (EIP), a thromboxane A2 receptor antagonist on platelet aggregation induced by arachidonic acid (AA, 0.4 mM) in platelet-rich plasma from a responder and a non-responder subject. In both specimens dazoxiben inhibited immunoreactive thromboxane B2 generation by more than 95%. The comparable inhibitory effect of EIP on platelet aggregation suggested a normal thromboxane A2 receptor function in both the responder and the non-responder subject. (From ref.[46], with permission.)

conducted on patients requiring low daily doses of warfarin[40]. The frequency of the two allelic variants of CYP2C9 polymorphism in this population has been compared to that of a control group of patients with a wide range of dose requirements attending an anticoagulation clinic and to a control group of healthy volunteers. A strong association has been found between the need for a low dose of warfarin and the presence of at least one mutant allele of the CYP2D9 gene. Moreover, in these patients the induction of anticoagulation was more difficult than in controls, as indicated by longer hospitalization or repeated visits to the outpatient clinic. The lower metabolism of warfarin also makes carriers several times more susceptible to bleeding complications in spite of low dose administration. The concern for this complication is justified by the high mortality risk, especially in the aged population. Therefore, identification of these polymorphisms before starting the therapy might help prevent complications.

The recent discovery of different mutations in coagulation Factor IX has revealed a new type of genetic predisposition to bleeding complications during oral anticoagulant therapy. When receiving warfarin or other coumarins, patients with these variants show a very marked decrease in Factor IX activity approaching that of severe hemophilia B. As a consequence these patients may suffer from bleeding at the beginning of therapy, even before the therapeutic range has been reached[41].

The cost of large-scale genetic screening for different responsiveness to warfarin might be quite high (at least in the forthcoming years), but it should be verified and weighed against prolonged hospitalization, emergency admission for bleeding complications and mortality risk[42].

Responders and non responders to Tx-synthase inhibitors: an *antelitteram* example of pharmacogenetics

As already mentioned, the fascinating concept that selective Tx-synthase inhibition might suppress platelet function while leaving intact vascular prostacyclin generation was developed in the early 1980s[43,44].

However, several studies showed that, when preincubated with a Tx-synthase inhibitor, only the platelets from some subjects (so-called responders) were inhibited, i.e. they did not aggregate after arachidonic acid stimulation, whereas platelets from other subjects (non responders) continued to aggregate despite a similar degree of suppression of TxA2 formation[2,45–50] (Fig. 65.1). It was suggested that in some particular individuals the complex balance between aggregatory (PG endoperoxides, PGE2) and inhibitory (PGD2) prostaglandins and their interactions with specific receptors might result in prevention or stimulation of platelet aggregation associated with TxA2 synthesis inhibition[2].

The unequal functional response of platelets from different subjects to Tx-synthase inhibition was shown to depend on adenylate cyclase function[49,50], that modulates the inhibitory activity of PGD2[51].

Some authors found it not easy to identify characteristics that clearly distinguished platelets from responders and non-responders to Tx-synthase inhibition. Platelets from responders and non responders appeared equally sensitive to the aggregating effect of U46619, an endoperoxide analogue and a TxA2 mimic; the amount of PGE2 generated in the presence of a Tx-synthase inhibitor was very similar in responder and non-responder platelets and they showed the same sensitivity to inhibition of platelet aggregation by PGD2[2,45,48].

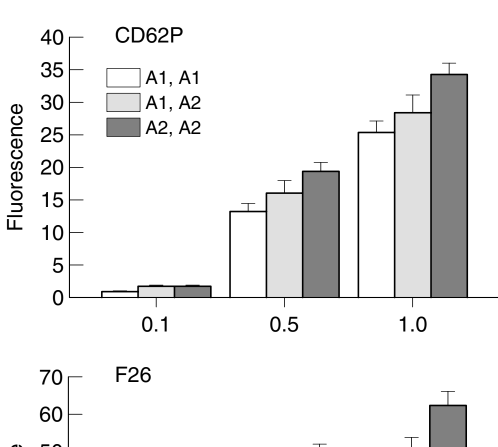
Gresele et al.[50], however, found that higher amounts of PGD2, prostacyclin and adenosine were required to suppress arachidonic acid-induced aggregation of platelets from non-responders, while they observed no difference between responders and non-responders concerning platelet sensitivity to forskolin or dibutyryl cyclic AMP. The

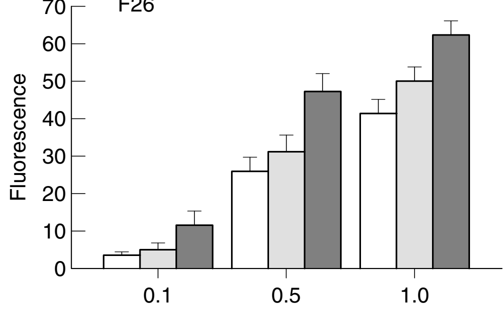
Cambridge Books Online © Cambridge University Press, 2009

Table 65.2. Platelet aggregation induced by epinephrine and ADP in relation to platelet GPIIIa Pl^A polymorphism

	Genotype			
Platelet agonist	$Pl^{A1/A1}$	$Pl^{A1/A2}$	$Pl^{A2/A2}$	P^a
Epinephrine (μM)	0.9 (0.9–1.0)	0.7 (0.7–0.9)	0.6 (0.4–1.0)	0.007
ADP (μM)	3.1 (3.0–3.2)	3.0 (2.9–3.2)	2.8 (2.4–3.3)	0.190

Note:
[a] P values are ANOVA, adjusted for age, sex, BMI, diabetes, triglyceride, total and HDL cholesterol, cardiovascular disease, menopausal status, and estrogen replacement therapy.
Source: From ref.[56], with permission.





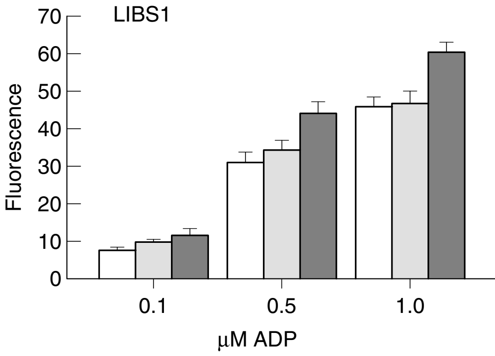


Fig. 65.2. Platelet activation state in whole blood from normal donors, carriers of Pl^{A1} and Pl^{A2} genotypes, stimulated with low-dose ADP. Flow cytometric analysis of surface expression of P-selectin (CD62P), GPIIb–IIIa-bound fibrinogen (F26) and activated GPIIb–IIIa (LIBS1). (From ref.[57], with permission.)

cyclic AMP rise induced by prostacyclin was lower in platelets from non-responders; exogenous PGE2 exerted a pro-aggregatory effect on platelets from all non-responders, but not in platelets from most of the responders. These authors suggested the reason for the relative insensitivity of non-responder platelets to adenylate cyclase stimulators might be an intrinsic hereditary defect of the enzyme[50].

Whether the observation of the existence of responders and non-responders to Tx-synthase inhibitors might be considered as an early unrecognized example of platelet pharmacogenetics is a stimulating possibility, which, however, lacks any direct proof.

Polymorphisms of platelet glycoprotein IIIa and the efficacy of abciximab and aspirin

The glycoprotein IIb–IIIa complex acts as a receptor for fibrinogen and other adhesive molecules on the surface of platelets and is required for their aggregation[10,11]. Polymorphism PI^{A2} is a variant of the gene encoding for glycoprotein IIIa and is present in about 25% of the white population[52]. This polymorphism has been associated with an overall moderate, but significant increased risk of coronary heart disease, especially at young age and in patients undergoing coronary bypass[52–54]. It has recently been shown that PI^{A2} allele is associated with an increased reactivity of platelets to aggregation and with an altered integrin-mediated function of adhesion, spreading and clot retraction[55]. Within the large Framingham Offspring Study, GPIIIa genotype and platelet reactivity phenotype was determined in 1,422 subjects. Allele frequencies of Pl^{A1} and Pl^{A2} were 0.84 and 0.16, respectively. The presence of one or both Pl^{A2} alleles was associated with increased platelet aggregability by adrenaline and, to a lesser extent, by ADP (Table 65.2)[56].

Michelson et al.[57] studied 56 normal donors and compared platelet function in Pl^{A1} and Pl^{A2} genotype carriers: compared with homozygous Pl^{A1} platelets, Pl^{A2} positive platelets showed a significantly higher surface-expressed P-selectin, GPIIb–IIIa-bound fibrinogen and activated GPIIb–IIIa in response to low concentrations of ADP (Fig. 65.2). Surface expression of GPIIb–IIIa was similar in resting platelets of all three genotypes but was significantly greater on $Pl^{A2/A2}$ platelets after ADP stimulation.

While the observations mentioned above offer a biological plausibility to the thrombogenic effect of the Pl^{A2} polymorphism[54], other studies seem to exclude its relation

Cambridge Books Online © Cambridge University Press, 2009

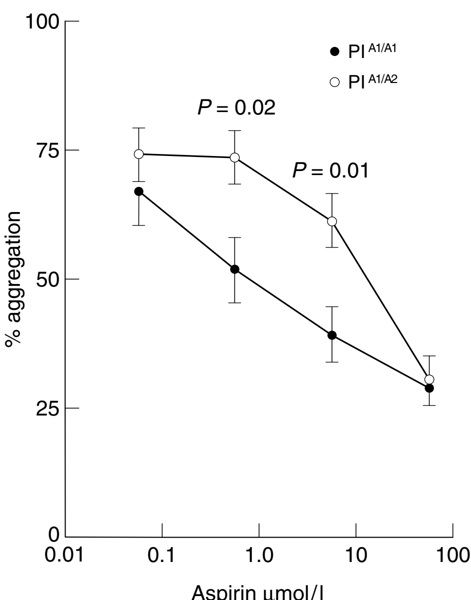


Fig. 65.3. Inhibition of aggregation of $Pl^{A1/A1}$ and $Pl^{A1/A2}$ platelets by aspirin. (From ref.[60], with permission.)

to platelet hyper-reactivity: indeed hyporeactivity of platelets from carriers of Pl^{A2} polymorphism was reported when thrombin or ADP[58], and arachidonic acid or a TxA2 analogue[59] were used as agonists.

In a way unrelated to its possible effect on platelet reactivity to aggregating stimuli, the presence of PI^{A2} polymorphism might influence the antiaggregatory effect of platelet inhibitory drugs[57,60]. In particular, abciximab (ReoPro), a monovalent chimeric Fab fragment of the 7E3 antibody that blocks ligand binding to GPIIb–IIIa and is reportedly beneficial in coronary ischemic syndromes[11], caused small but significant differences among genotypes in inhibiting aggregation. The IC50 (μg/ml) for abciximab was 1.90 ± 0.21 in platelets with the $Pl^{A1/A1}$ genotype but 2.27 ± 0.19 in platelets with $Pl^{A1/A2}$ genotype and 2.13 ± 0.14 in those with $Pl^{A2/A2}$ genotype. Similarly, there were significant differences among these genotypes in the aspirin IC50 (μM): (7.4 ± 2.5 vs. 13.1 ± 3.7 and 14.0 ± 2.1, respectively)[57].

In an earlier study by the same group[60], the inhibitory efficacy of aspirin had been tested in vitro against adrenaline-induced platelet aggregation. The IC50 (μM) of aspirin was 22.8 ± 5.8 in $Pl^{A1/A1}$ platelets but only 2.3 ± 1.2 (a tenfold difference) in $Pl^{A1/A2}$ platelets (Fig. 65.3).

An increased sensitivity to the inhibitory effect of aspirin was also observed in platelets challenged with arachidonic acid from subjects with the Pl^{A2} allele[59].

These in vitro data are to be interpreted in relation to in vivo experiments. Undas et al.[61], measuring the amount of thrombin generated in blood samples collected from bleeding time wounds, found that aspirin (75 mg/daily for 7 days) was inhibitory in 23 out of 25 $Pl^{A1/A1}$ but only in 9 out of 15 $Pl^{A1/A2}$ carriers ($P = 0.02$). Average thrombin generation decreased in A1 homozygous by 63% but in A2 carriers by only 28%. The same group[62] has lately reported that bleeding time was significantly shorter in carriers of the Pl^{A2} allele than in Pl^{A1} homozygous. Four hours after ingestion of 300 mg aspirin, the difference between the two groups was increased: in $Pl^{A1/A1}$ the average bleeding time was prolonged from 372 s to 485 s (Δ 113 s), while in the $Pl^{A1/A2}/Pl^{A2/A2}$ group it was only prolonged from 302 s to 349 s (Δ 47 s). In the latter group, bleeding time after aspirin was still shorter than bleeding time before aspirin in the former group (349 s vs. 372 s).

Altogether, these data support the hypothesis that differential platelet sensitivity to commonly used agents could influence the response of patients to these drugs. However, which aspect of in vitro and/or in vivo platelet function would better reflect the genetic control of the antithrombotic action of antiplatelet drugs remains to be established.

It has been suggested that receptor clustering, a phenomenon that increases GPIIb–IIIa signalling[63], might be inhibited in carriers of a given polymorphism but not of another, so that platelets become more or less susceptible to inhibition by antiplatelet drugs. The significant differences in inhibition may affect either beneficial (antithrombotic) or adverse (hemorrhagic) effects. The GPIIb–IIIa is the most abundant receptor on human blood platelets (about 80000 copies per platelet or 12×10^{16} copies in our body), so that even a subtle functional alteration could have profound effects over the lifetime of a patient[57].

Pharmacogenetics: a new key of interpretation of the Thrombosis Prevention Trial

Analyzing the results of the Thrombosis Prevention Trial[16] in the light of a genetic approach to pharmacology, new interpretations for drug efficacy and new clinical trial designs have been proposed[64]. This trial was performed by general practitioners in the United Kingdom and aimed at finding differences in a primary prevention approach of high risk individuals for cardiovascular disease, by treating them at random with low dose warfarin and/or low dose aspirin against placebo. The prospective follow-up revealed that the group receiving warfarin had the same quantitative reduction in all ischemic events as did the group receiving aspirin. The beneficial effect was greater

Cambridge Books Online © Cambridge University Press, 2009

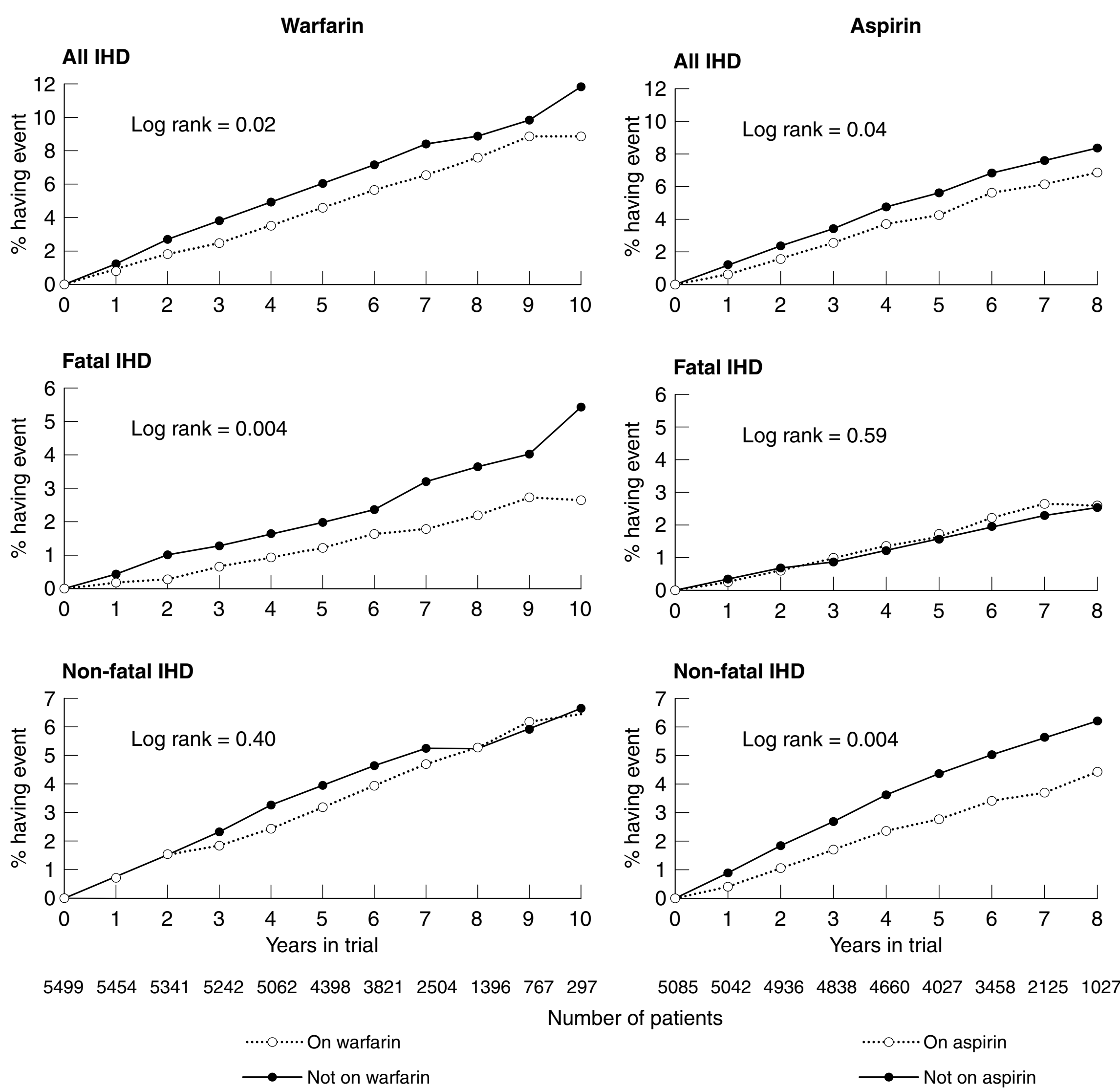


Fig. 65.4. Cumulative proportion (percent) of men with ischemic heart disease (IHD), main effects. *n*, number of patients still on trial for specified duration of follow-up. (From ref.[16], with permission.)

for the group receiving the combined therapy (Fig. 65.4). Bleeding being still a problem of anticoagulant and antiplatelet therapy, subjects receiving the combination also showed an increased hemorrhagic risk as compared to placebo and individual therapies. Future trials might consider genetic categories related to platelet function and coagulation factor activity as a model to predict drug efficacy and risk of bleeding of patients, ultimately providing the choice of the best antithrombotic drug for each patient or subject. In this context, we have already mentioned the Pl^A polymorphisms which may render platelets more or less sensitive to aspirin[57–62]. Polymorphisms in coagulation factor VII and their effect in reducing factor VII levels and the risk of myocardial infarction have also been described[65,66].

On the basis of these considerations, the population included in the Thrombosis Prevention Trial might be theoretically divided into four groups according to the polymorphisms $Pl^{A1/A2}$ of platelet GPIIIa and R356Q of factor VII gene (Table 65.3). The first group includes subjects carrying the alleles Pl^{A2} and 353Q. The second one carries the allele 353R combined with Pl^{A1}. The third group includes carriers of factor VII allele 353R but combined with Pl^{A2}. Finally, the fourth group consists of factor VII allele 353Q carriers

Cambridge Books Online © Cambridge University Press, 2009

Table 65.3. Simulation of results of the Thrombosis Prevention Trial[16] if subjects were categorized according to platelet glycoprotein (GP) IIIa Pl$^{A1/A2}$ [57,60] and R353Q factor VII (FVII)[65] polymorphisms

GPIIIa Pl$^{A1/A2}$	Platelet	R353Q allele	FVII levels	Warfarin	Aspirin
A2	↑	Q	LOW	−	+
A1	↓	R	HIGH	+	−
A2	↑	R	HIGH	+	+
A1	↓	Q	LOW	−	−

Note:
↑ increased aggregability and higher sensitivity to aspirin; ↓ decreased aggregability and lower sensitivity to aspirin; + therapeutical benefit; − no therapeutical benefit.
Source: From ref.[31], with permission.

combined with Pl^{A1} allele of GPIIIa. According to the associations genotype/phenotype described in the literature, these groups might have: increased platelet aggregability, high sensitivity to aspirin and low factor VII levels (first group); decreased platelet function, low sensitivity to aspirin and high factor VII levels (second group); increased platelet function, high sensitivity to aspirin and high factor VII levels (third group); and finally decreased platelet function, low sensitivity to aspirin and low factor VII levels (fourth group). We could expect that the first group would benefit from aspirin. It would be useful to reduce high platelet reactivity with a drug to which platelets are genetically sensitive; moreover further reduction by warfarin of already genetically low levels of factor VII could only expose patients to hemorrhagic side effects. In a similar way, the second group should benefit from warfarin, the third group from the combination of aspirin and warfarin, and finally the fourth group should not benefit either from aspirin or from warfarin. The latter could be an ideal group for testing new antithrombotic drugs.

We have also mentioned the CYP2C9 gene polymorphism which can distinguish 'poor' and 'high' metabolizers of warfarin[40]: the former patients should be identified and monitored with particular care as they would be preferential candidates to hemorrhagic side effects.

Metabolism of thienopyridine prodrugs to exhibit antiaggregating activity and/or side effects

Ticlopidine and clopidogrel need to be administered in vivo to exhibit antiaggregating activity. Although some direct effects of thienopyridines in vitro have been reported, most of them are observed at non-relevant doses and do not seem to account for the antiaggregatory activity of these drugs, responsible for their antithrombotic properties[9]. The achievement of an antiaggregating effect only after in vivo administration suggests that the thienopyridines do not act directly on platelets, and shows that active antiaggregating substance(s) must be produced through a metabolic process[9]. A study performed on clopidogrel showed the liver was the metabolic site from which the antiaggregant activity of this drug originates. The hepatic bioactivation of clopidogrel required a cytochrome P450–1A (CYP-1A)-dependent metabolism[8].

A study of the metabolism of ticlopidine resulted in the identification of about 20 separate metabolites, eight representing approximately 30% of the initial compound, but none of them had in vitro activity. The other metabolites representing approximately 70% of the initial compound have not yet been identified, but no study has been able to demonstrate an antiaggregant activity in the plasma of treated subjects[67,68]. This suggests that the active metabolite(s) circulate at very low concentrations and/or may have a very short half-life/lives. Furthermore, the platelets of clopidogrel-treated subjects remain resistant to ADP even after washing, in vitro[67,68] or after the end of treatment, and the rate at which aggregation is restored correlates closely with platelet production[69]. These observations suggest the presence of an active metabolite of clopidogrel, produced by the liver, acting in an irreversible manner on platelets. This compound has recently been purified and its chemical structure determined[8,9]. It is a thiol reactive of clopidogrel which directly targets the ADP- receptor binding on platelets. This interaction is highly specific and irreversible, two features which correspond to the antiaggregating activity observed after clopidogrel treatment. Similarly to what has already been shown for CYP2C9-dependent metabolism of warfarin[40], it is likely that the metabolism of ticlopidine and clopidogrel[70] is also genetically modulated, f.i. by polymorphisms of the P-450–1A cytochrome in the liver (Fig. 65.5).

Not only the efficacy of these drugs but also their safety could be better monitored by a pharmacogenetic approach. Although no cases of thrombotic thrombocytopenic purpura (TTP) were reported among the 20 000 patients who received clopidogrel in clinical trials[7], 11 cases of this serious hematologic adverse effect have been recently described that occurred in patients during or shortly after they received the drug[71]. Nine additional cases of clopidogrel-associated TTP were identified in the weeks following Bennett et al.'s paper[72]. The disorder began within 14 days after the initiation of clopidogrel therapy in all but one patient.

Cambridge Books Online © Cambridge University Press, 2009

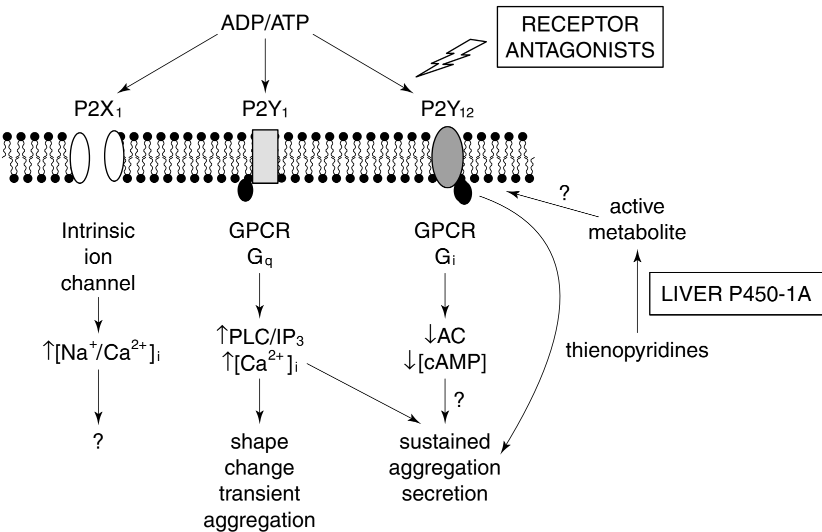


Fig. 65.5. The three-receptor model of ADP-induced platelet activation indicating the sites of action of receptor antagonists and of thienopyridines and their liver metabolite(s). (Modified from ref.[70], with permission.)

In several countries the occurrence of side effects after a drug is marketed is monitored by voluntary reporting systems. However, it has been calculated that only 1% to 10% of all side effects is reported to a reporting body such as the Food and Drug Administration (FDA)-controlled system MedWatch[73]. The need for new, non-traditional approaches to drug safety has been underlined in the Editorial accompanying the report of TTP-clopidogrel association[73], but no mention is made of the possibility that people could be genetically predisposed to develop a side effect while using ticlopidine or clopidogrel. An immune-mediated deficiency of von Willebrand factor-cleaving metalloproteinase activity has been reported in some patients with thienopiridine-related TTP[71,74], thus a mutation or a polymorphism of this protease might contribute to the development of the clinical syndrome.

A novel platelet ADP receptor and its polymorphisms

Hollopeter et al.[75] have recently described a novel ADP receptor expressed in platelets that mediates platelet aggregation via inhibition of adenylyl cyclase. They identified and cloned the P2Y12 receptor, and showed that it is abundantly expressed on the surface of human platelets, and to a smaller extent in the brain. The predominant transcript of P2Y12 was absent from all other tissues examined. The gene for P2Y12 is located on chromosome 3, in a region that contains genes encoding at least three receptors, two of which mediate ADP-dependent platelet aggregation. Analysis of platelets from a patient previously diagnosed with a mild bleeding disorder revealed the presence of a mutant allele at the locus corresponding with the P2Y12 gene[76]. Although only one allele was affected by a frame-shift mutation, this patient did not produce any wild-type P2Y12 protein, suggesting that expression of the normal allele is repressed. As already mentioned, ticlopidine and clopidogrel block the P2Y12 receptor, but have therapeutic limitations. These include their slow onset of action, irreversible blockade of the receptor and undesirable side effects in some patients. Now that the receptor has been cloned, the development of more highly targeted drugs should be accelerated. Some groups are also developing a P2Y12-deficient mouse to further evaluate the role of this receptor in normal platelet function and in thrombotic models of vascular injury. The more we know about this receptor, the more opportunities there will be for rational drug design. Future work to identify further mutations and polymorphisms in the receptor gene will be another important step. If abnormalities in P2Y12 are common, this could be a modulating factor in the population that can decrease risk for thrombotic disease[77–79].

A hypothetical case in 2010

General visions of gene-based medicine in the future are useful, and may allow to anticipate some future scenarios of the daily practice of medicine in a primary care setting. A hypothetical clinical encounter in 2010 has been recently

Cambridge Books Online © Cambridge University Press, 2009

described[80] and will be reported here with some modifications.

John, a 23-year-old college graduate, is referred to his physician because a serum cholesterol level of 255 mg per decilitre was detected in the course of a medical examination required for employment. He is in good health but has smoked one pack of cigarettes per day for 6 years. Aided by an interactive computer program that takes John's family history, his physician notes that there is a strong paternal history of myocardial infarction and that John's father died at the age of 48 years.

To obtain more precise information about his risks of contracting coronary artery disease and other illnesses in the future, John agrees to consider a battery of genetic tests. After working through an interactive computer program that explains the benefits and risks of such tests, John agrees (and signs informed consent) to undergo 15 genetic tests that provide risk information for illnesses for which preventive strategies are available. He decides against an additional ten tests involving disorders for which no clinically validated preventive interventions are yet available.

A cheek-swab DNA specimen is sent off for testing, and the results are returned in one week. John's subsequent counselling session with the physician and a genetic nurse specialist focuses on the conditions for which his risk differs substantially (by a factor of more than two) from that of the general population.

John is pleased to learn that genetic testing does not always give bad news. His risks of contracting prostate cancer and Alzheimer's disease are reduced, because he carries low-risk variants of the several genes known (in 2010) to contribute to these illnesses. But, John is sobered by the evidence of his increased risks of contracting coronary artery disease, colon cancer and lung cancer. Confronted with the reality of his own genetic data, he decides to introduce some change in health-related behaviour, focused on reducing specific risks. By 2010, the field of pharmacogenetics has blossomed, and a prophylactic drug regimen based on the knowledge of John's personal genetic data can be precisely prescribed to reduce not only his cholesterol level but his global risk of coronary artery disease to normal levels. In particular, as he carries the platelet glycoprotein IIIa Pl^{A2} variant gene, that makes platelets more sensitive to aspirin inhibition[57,59] a low-dose, slow release, bone marrow targeted aspirin is prescribed (to be taken twice a week). On the other hand, considering that John carries the B2B2 polymorphism of the cholesterol ester transfer protein (CETP), that is present in about 16% of the population and makes its carriers quite resistant to cholesterol lowering drugs[81] no prevention with a statin is planned. John also carries the ApoEe4 form of the ApoE polymorphism, that shows a high response to probucol, another cholesterol lowering drug[82]; thus he is given dietary advice and a probucol course. John is also informed to be a genetically 'slow' ethanol oxidizer as he is homozygous for the alcohol dehydrogenase type 3 $\gamma 2$ allele: in moderate wine drinkers (one or two drinks per day) this allele is associated not only with a reduced rate of ethanol oxidation, but also with high plasma levels of HDL and a substantially decreased risk of myocardial infarction (relative risk: 0.14, 95% confidence limits from 0.04 to 0.45, in respect to carriers of $\gamma 1$ $\gamma 1$ alleles who consume less than one drink per week)[83]. Although individuals with a slow rate of metabolism of ethanol may be at higher risk for alcohol-associated diseases, John, who is already a usual moderate wine consumer, is encouraged to continue this dietary habit and to drink with meals[84]. His risk of colon cancer will be addressed by beginning a programme of annual colonoscopy at the age of 45, which in his situation is a very cost-effective way to avoid colon cancer. His substantial risk of contracting lung cancer provides the key motivation for him to join a support group of persons at genetically high risk for serious complications of smoking, and he successfully kicks the habit.

Next stop: proteomics

The simplified, though useful, idea of one gene to one protein to one function is no more acceptable, as cells and organisms are far more complex than is indicated by analysis of the genetic material alone. The concept of a human protein index was put forward in the 1980s[85]: proteins are the functional output of the cell and might therefore be expected to provide the most relevant information in specific biological contexts[86]. Proteomics thus complements the genomics, as the genetic code cannot always indicate which proteins are expressed, if they are glycosylated or phosphorylated, which is their three-dimensional structure, and finally, what is (are) their function(s). Moreover, the influence of environmental factors such as dietary habits or drug administration or of multifactorial processes such as ageing, cardiovascular disease or cancer cannot be fully evaluated by the simple analysis of the genome. Therefore, new perspectives and new biomedical opportunities are emerging by proteomics-based approaches[86]). Preliminary studies suggest an average number of three to more than six protein forms per each gene, in human beings[87]. This means that the human body may contain more than 200 000 modified proteins. Most drugs, including antiplatelet agents, exert their effects on

Cambridge Books Online © Cambridge University Press, 2009

proteins. It is thus conceivable that in the postgenome era a concerted strategy with proteomics approaches will allow pharmacogenetics to achieve its potential in biomedical research and clinical practice.

Acknowledgements

The Authors wish to thank Maria Benedetta Donati for continued discussions and encouragement and Attilio Maseri for his inspiring criticism to the current clinical megatrial approach. Alexandra Cianci helped prepare the figures. Special thanks and appreciation are expressed to Cleo Colombo for her competent, faithful and discreet secretarial work during the last 14 years.

References

1 de Gaetano G, Bertelé V, Cerletti C. Pharmacology of antiplatelet drugs. In MacIntyre DE, Gordon JL, eds. *Platelets in biology and pathology* III. Amsterdam: Elsevier; 1987: 515–573.
2 Bertelé V, Falanga A, Tomasiak M, Chiabrando C, Cerletti C, de Gaetano G. Pharmacologic inhibition of thromboxane synthetase and platelet aggregation: modulatory role of cyclooxygenase products. *Blood* 1984; 63: 1460–1466.
3 Gresele P, Deckmyn H, Nenci GG, Vermylen J. Thromboxane synthase inhibitors, thromboxane receptor antagonists and dual blockers in thrombotic disorders. *Trends Pharmacol Sci* 1991; 12: 158–163.
4 Praticò D, Cheng Y, FitzGerald GA. TP or not TP: primary mediators in a close runoff? *Arterioscler Thromb Vasc Biol* 2000; 20: 1695–1698.
5 Di Minno G, Cerbone AM, Mattioli PL, Turco S, Iovine C, Mancini M. Functionally thrombasthenic state in normal platelets following the administration of ticlopidine. *J Clin Invest* 1985; 75: 328–338.
6 Cattaneo M, Gachet C. ADP receptors and clinical bleeding disorders. *Arterioscler Thromb Vasc Biol* 1999; 19: 2281–2285.
7 CAPRIE Steering Committee. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). *Lancet* 1996; 48: 1329–1339.
8 Savi P, Combalbert J, Gaich C et al. The antiaggregating activity of clopidogrel is due to a metabolic activation by the hepatic cytochrome P450–1A. *Thromb Haemost* 1994; 72: 313–317.
9 Savi P, Herbert JM. Pharmacology of ticlopidine and clopidogrel. *Haematologica* 2000; 85 Suppl: 73–77.
10 Coller BS, Seligsohn U, Peretz H, Newman PJ. Glanzman thrombasthenia: new insights from an historical perspective. *Semin Hematol* 1994; 31: 301–311.
11 Coller BS. Binding of abciximab to alpha V beta 3 and activated alpha M beta 2 receptors: with a review of platelet-leukocyte interactions. *Thromb Haemost* 1999; 82: 326–336.
12 Verstraete M. Synthetic inhibitors of platelet glycoprotein IIb/IIIa in clinical development. *Circulation* 2000; 102: e76-e80.
13 Chew DP, Bhatt DL, Sapp S, Topol EJ. Increased mortality with oral platelet Glycoprotein IIb/IIIa antagonists: A Meta-Analysis of phase III multicenter randomized trials. *Circulation* 2001; 103: 201–206.
14 Culasso DE, Donati MB, de Gaetano G, Vermylen J, Verstraete M. Inhibition of human platelet aggregation by plasmin digests of human and bovine fibrinogen preparations: role of contaminating factor VIII-related material. *Blood* 1974; 44: 169–175.
15 ISIS-2 (Second International Study of Infarct Survival) Collaborative Group. Randomised trial of intravenous streptokinase, oral aspirin, both, or neither among 17,187 cases of suspected acute myocardial infarction: ISIS-2. *Lancet* 1988; ii: 349–360.
16 The Medical Research Council's General Practice Research Framework. Thrombosis prevention trial: randomised trial of low-intensity oral anticoagulation with warfarin and low-dose aspirin in the primary prevention of ischaemic heart disease in men at increased risk. *Lancet* 1998; 351: 233–241.
17 Collaborative Group of the Primary Prevention Project (PPP): Roncaglioni MC, Avanzini F, Colombo F et al. Low-dose aspirin and vitamin E in people at cardiovascular risk: a randomized trial in general practice. *Lancet* 2001; 357: 89–95.
18 Antiplatelet Trialists' Collaboration. Collaborative overview of randomised trials of antiplatelet therapy – I: Prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. *BMJ* 1994; 308: 81–106.
19 de Gaetano G. Aspirin and the prevention of ischaemic heart disease. A socratic dialogue between a cardiologist, a clinical pharmacologist and an expert of blood platelets. *Ital Heart J* 2001; 2: 582–583.
20 Steering Committee of the Physicians' Health Study Research Group. Final report on the aspirin component of the ongoing Physicians' Health Study. *N Engl J Med* 1989; 321: 129–135.
21 Liuzzo G, Biasucci LM, Gallimore JR et al. The prognostic value of C-reactive protein and serum amyloid a protein in severe unstable angina. *N Engl J Med* 1994; 331: 417–424.
22 Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med* 1997; 336: 973–979.
23 Maseri A. Antibiotics for acute coronary syndromes: are we ready for megatrials? *Eur Heart J* 1999; 20: 89–92.
24 Meade TW, Brennan PJ. Determination of who may derive most benefit from aspirin in primary prevention: subgroup results from a randomised controlled trial. *BMJ* 2000; 321: 13–17.
25 Dyer MR, Cohen D, Herrling PL. Building a competitive functional genomics platform. *Nature Biotechnol* 1999; 17 Suppl: 18–19.
26 Anderson W, Fitzgerald C, Manasco P. Current and future applications of pharmacogenomics. *New Horiz* 1999; 7: 262–269.

Cambridge Books Online © Cambridge University Press, 2009

27 Dyer MR, Cohen D, Herrling PL. Functional genomics: from genes to new therapies. *Drug Discov Today* 1999; 4: 109–114.
28 Gurwitz D. Pharmacogenetic-oriented drug development. *Drug Discov Today* 1999; 4: 151–152.
29 Ledley F. Can pharmacogenomics make a difference in drug development? *Nature Biotechnol* 1999; 17: 731.
30 Sadee's W. Pharmacogenomics. *BMJ* 1999; 319: 1286.
31 Dirckx C, Donati MB, Iacoviello L. Pharmacogenetics: a molecular sophistication or a new clinical tool for cardiologists? *Ital Heart J* 2000; 1: 662–666.
32 Meyer UA. Pharmacogenetics and adverse drug reactions. *Lancet* 2000; 356, 1667–1671.
33 Pfost DR, Boyce-Jacino MT, Grant DM. A SNPshot: pharmacogenetics and the future of drug therapy. *Trends Biotechnol* 2000; 18: 334–338.
34 Roses AD. Pharmacogenetics and future drug development and delivery. *Lancet* 2000; 355: 1358–1361.
35 Roses AD. Pharmacogenetics and the practice of medicine. *Nature* 2000; 405: 857–865.
36 Wolf CR, Smith G, Smith RL. Science, medicine, and the future: pharmacogenetics. *BMJ* 2000; 320: 987–990.
37 Weiss KM, Terwilliger JD. How many diseases does it take to map a gene with SNPs? *Nat Genet* 2000; 26: 151–157.
38 The European Agency for the Evaluation of Medicinal products. A Seminar on the use of pharmacogenetics in the drug development process. Emea, 2000, The Colour Works Printing Co, Ltd. England: pp 1–36.
39 Weber WW. Populations and genetic polymorphisms. *Mol Diagn* 1999; 4: 299–307.
40 Aithal GP, Day CP, Kesteven PJ, Daly AK. Association of polymorphisms in the cytochrome P450 CYP2C9 with warfarin dose requirement and risk of bleeding complications. *Lancet* 1999; 353: 717–719.
41 Oldenburg J, Kriz K, Wuillemin WA et al. for the Study Group on Hereditary Warfarin Sensitivity. Genetic predisposition to bleeding during oral anticoagulant therapy: evidence for Common Founder Mutations (FIXVal-10 and FIXThr-10) and an independent CpG Hotspot Mutation (FIXThr-10). *Thromb Haemost* 2001; 85: 454–457.
42 Mannucci PM. Genetic control of anticoagulation. *Lancet* 1999; 353: 688–689.
43 Vermylen J, Defreyn G, Carreras LO, Machin SJ, Van Schaeren J, Verstraete M. Thromboxane synthetase inhibition as antithrombotic strategy. *Lancet* 1981; i: 1073–1075.
44 Tyler HM, Saxton CA, Parry MJ. Administration to man of UK-37,248–01, a selective inhibitor of thromboxane synthetase. *Lancet* 1981; 1: 629–632.
45 Heptinstall S, Bevan J, Cockbill SR, Hanley SP, Parry MJ. Effects of a selective inhibitor of thromboxane synthetase on human blood platelet behaviour. *Thromb Res* 1980; 20: 219–230.
46 Bertelé V, Cerletti C, Schieppati A, Di Minno G, de Gaetano G. Inhibition of thromboxane synthetase does not necessarily prevent platelet aggregation. *Lancet* 1981; i: 1057–1058.
47 Gresele P, Deckmyn H, Huybrechts E, Vermylen J. Serum albumin enhances the impairment of platelet aggregation with thromboxane synthase inhibition by increasing the formation of prostaglandin D2. *Biochem Pharmacol* 1984; 33: 2083–2088.
48 Rajtar G, Cerletti C, Castagnoli MN, Bertelé V, de Gaetano G. Prostaglandins and human platelet aggregation. Implications for the anti-aggregating activity of thromboxane-synthase inhibitors. *Biochem Pharmacol* 1985; 34: 307–310.
49 Bertelé V, Falanga A, Tomasiak M, Cerletti C, de Gaetano G. SQ 225, an adenylate cyclase inhibitor, prevents the antiplatelet effect of dazoxiben, a thromboxane synthase inhibitor. *Thromb Haemost* 1984; 51: 125–128.
50 Gresele P, Blockmans D, Deckmyn H, Vermylen J. Adenylate cyclase activation determines the effect of thromboxane synthase inhibitors on platelet aggregation in vitro. Comparison of platelets from responders and nonresponders. *J Pharmacol Exp Ther* 1988; 246: 301–307.
51 Mills DC, Macfarlane DE. Stimulation of human platelet adenylate cyclase by prostaglandin D2. *Thromb Res* 1974; 5: 401–412.
52 Weiss EJ, Bray PF, Tayback M et al. A polymorphism of a platelet glycoprotein receptor as an inherited risk factor for coronary thrombosis. *N Engl J Med* 1996; 334: 1090–1094.
53 Laule M, Cascorbi I, Stangl V, Bielecke C, Wernecke KD, Mrozikiewicz PM, Felix SB, Roots I, Baumann G, Stangl K. A1/A2 polymorphism of glycoprotein IIIa and association with excess procedural risk for coronary catheter interventions: a case-controlled study. *Lancet* 1999; 353: 708–772.
54 Di Castelnuovo A, de Gaetano G, Donati MB, Iacoviello L. Platelet glycoprotein receptor IIIa polymorphism PlA1/PlA2 and vascular risk: a meta-analysis. *Thromb Haemost* 2001; 85: 626–633.
55 Vijayan KV, Goldschmidt-Clermont PJ, Roos C, Bray PF. The Pl(A2) polymorphism of integrin beta(3) enhances outside-in signalling and adhesive functions. *J Clin Invest* 2000; 105: 793–802.
56 Feng D, Lindpaintner K, Larson MG et al. Increased platelet aggregability associated with platelet GPIIIa PlA2 polymorphism: the Framingham Offspring Study. *Arterioscler Thromb Vasc Biol* 1999; 19: 1142–1147.
57 Michelson AD, Furman MI, Goldschmidt-Clermont P et al. Platelet GPIIIa PlA polymorphisms display different sensitivities to agonists. *Circulation* 2000; 101: 1013–1018.
58 Lasne D, Krenn M, Pingault V et al. Interdonor variability of platelet response to thrombin receptor activation: influence of PlA2 polymorphism. *Br J Haematol* 1997; 99: 801–807.
59 Andrioli G, Minuz P, Solero P, Pincelli S, Ortolani R, Lussignoli S, Bellavite P. Defective platelet response to arachidonic acid and thromboxane A(2) in subjects with Pl(A2) polymorphism of beta(3) subunit (glycoprotein IIIa). *Br J Haematol* 2000; 110: 911–918.
60 Cooke GE, Bray PF, Hamlington JD, Pham DM, Goldschmidt-Clermont PJ. PlA2 polymorphism and efficacy of aspirin. *Lancet* 1998; 351: 1253.
61 Undas A, Sanak M, Musial J, Szczeklik A. Platelet glycoprotein IIIa polymorphism, aspirin, and thrombin generation. *Lancet* 1999; 353: 982–983.
62 Szczeklik A, Undas A, Sanak M, Frolow M, Wegrzyn W.

Cambridge Books Online © Cambridge University Press, 2009

Relationship between bleeding time, aspirin and the PlA1/A2 polymorphism of platelet glycoprotein IIIa. *Br J Haematol* 2000; 110: 965–967.
63 Shattil SJ, Kashiwagi H, Pampori N. Integrin signalling: the platelet paradigm. *Blood* 1998; 91: 2645–2657.
64 Iacoviello L, Donati MB. Interpretation of Thrombosis Prevention Trial. *Lancet* 1998; 351: 1205.
65 Iacoviello L, Di Castelnuovo A, De Knijff P et al. Polymorphisms in the coagulation factor VII gene and the risk of myocardial infarction. *N Engl J Med* 1998; 338: 79–85.
66 Girelli D, Russo C, Ferraresi P et al. Polymorphisms in the factor VII gene and the risk of myocardial infarction in patients with coronary artery disease. *N Engl J Med* 2000; 343: 774–780.
67 Savi P, Heilmann E, Nurden P. Clopidogrel: an antithrombotic drug acting on the ADP-dependent activation pathway of human platelets. *Clin Appl Thromb Haemost* 1996; 2: 35–42.
68 Di Perri T, Pasini FL, Frigerio C. Pharmacodynamics of ticlopidine in man in relation to plasma and blood cell concentration. *Eur J Clin Pharmacol* 1991; 41: 429–434.
69 Panak E, Maffrand JP, Picard-Fraire C, Vallee E, Blanchard J, Roncucci R. Ticlopidine: a promise for the prevention and treatment of thrombosis and its complications. *Haemostasis* 1983; 13 Suppl 1: 1–54.
70 Humphries RG. Pharmacology of AR-C69931MX and related compounds: from pharmacological tools to clinical trials. *Haematologica* 2000; 85 Suppl: 66–72.
71 Bennett CL, Connors JM, Carwile JM et al. Thrombotic thrombocytopenic purpura associated with clopidogrel. *N Engl J Med* 2000; 342: 1773–1777.
72 Bennett CL, Connors JM. Clopidogrel and thrombotic thrombocytopenic purpura. Letter to the Editor. *N Engl J Med* 2000; 343: 1191.
73 Wood AJJ. Clopidogrel and thrombotic thrombocytopenic purpura – a need for new approaches to drug safety. Editorial. *N Engl J Med* 2000; 342: 1824–1826.
74 Tsai HM, Rice L, Sarode R, Chow TW, Moake JL. Antibody inhibitors to von Willebrand factor metalloproteinase and increased binding of von Willebrand factor to platelets in ticlopidine-associated thrombotic thrombocytopenic purpura. *Ann Intern Med* 2000; 132: 794–799.
75 Hollopeter G, Jantzen HM, Vincent D et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* 2001; 409: 202–207.
76 Nurden P, Savi P, Heilmann E et al. An inherited bleeding disorder linked to a defective interaction between ADP and its receptor on platelets. Its influence on glycoprotein IIb–IIIa complex function. *J Clin Invest* 1995; 95: 1612–1622.
77 Cattaneo M, Lecchi A, Randi AM, McGregor JL, Mannucci PM. Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. *Blood* 1992; 80: 2787–2796.
78 Oury C, Toth-Zsamboki E, Van Geet C et al. A natural dominant negative P2X1 receptor due to deletion of a single amino acid residue. *J Biol Chem* 2000; 275: 22611–22614.
79 Senior K. Elusive platelet ADP receptor identified. Editorial. *Lancet* 2001; 357: 125.
80 Collins FS. Shattuck lecture – medical and societal consequences of the Human Genome Project. *N Engl J Med* 1999; 341: 28–37.
81 Kuivenhoven JA, Jukema JW, Zwinderman AH et al. The role of a common variant of the cholesteryl ester transfer protein gene in the progression of coronary atherosclerosis. The Regression Growth Evaluation Statin Study Group. *N Engl J Med* 1998; 338: 86–93.
82 Nestruck AC, Bouthillier D, Sing CF, Davignon J. Apolipoprotein E polymorphism and plasma cholesterol response to probucol. *Metabolism* 1987; 36: 743–747.
83 Hines LM, Stampfer MJ, Ma J et al. Genetic variation in alcohol dehydrogenase and the beneficial effect of moderate alcohol consumption on myocardial infarction. *N Engl J Med* 2001; 344: 549–555.
84 Rotondo S, Di Castelnuovo A, de Gaetano G. The relationship between wine consumption and cardiovascular risk: from epidemiological evidence to biological plausibility. *Ital Heart J* 2001; 2: 1–8.
85 Anderson NG, Anderson L. The Human Protein Index. *Clin Chem* 1982; 28: 739–748.
86 Banks RE, Dunn MJ, Hochstrasser DF, Sanchez JC, Blackstock W, Pappin DJ, Selby PJ. Proteomics: new perspectives, new biomedical opportunities. *Lancet* 2000; 356: 1749–1756.
87 Wilkins MR, Sanchez JC, Williams KL, Hochstrasser DF. Current challenges and future applications for protein maps and post-translational vector maps in proteome projects. *Electrophoresis* 1996; 17: 830–838.

Cambridge Books Online © Cambridge University Press, 2009

66

Cardiovascular gene therapy: implications for platelet vessel wall interactions

Pierre Zoldhelyi, Harold Eichstaedt, Thomas Jax, Janice M. McNatt, Zhi Qiang Chen, Harnath S. Shelat, Harris Rose and James T. Willerson

Wafic Said Molecular Cardiology and Gene Therapy Research Laboratory and Cullen Research Laboratory, Texas Heart Institute, and Department of Medicine (Divisions of Cardiology and of Clinical Pharmacology), The University of Texas Medical School at Houston, USA

Introduction

Evidence for the occurrence of coronary thrombosis early after percutaneous coronary interventions in humans was provided by serial angioscopic studies that demonstrated the development of varying degrees of thrombosis in over 90% of cases within 60 min after successful coronary balloon angioplasty[1]. Furthermore, studies on the effectiveness of acute PTCA during unstable angina have indicated not only an increased number of periprocedural complications but a relatively high incidence of restenosis[2,3]. Based on experimental and clinical evidence, it has been suggested that arterial thrombosis promotes the fibroproliferative response during later restenosis and progression of atherosclerosis[4–6]. The platelet IIb/IIIa and α_v/β_3 integrin receptor blocker, abciximab (ReoPro), reduced the need for recurrent clinical revascularization only in the EPIC trial[7], which enrolled patients with unstable angina (due, in general, to coronary thrombosis). In contrast, the later EPILOG trial in patients without acute coronary thrombosis failed to show a reduction in the need for recurrent revascularization[8], consistent with a role of early acute thrombosis in the pathogenesis of clinical restenosis. The incidence of periprocedural complications and 'clinically apparent' thrombosis after PTCA and stenting is sharply decreased by the administration of antiplatelet regimens, including the platelet IIb/IIIa receptor blockers and aspirin/ticlopidine or plavix. The efficacy of such interventions for the long-term prevention of 'subclinical' thrombosis and platelet deposition is uncertain, especially after these agents are withdrawn. Nevertheless, platelet thrombi are a rich source of vasoconstriction, mitogens, and chemotactic agents (Table 66.1 and Fig. 66.1).

Table 66.1. Selected thrombus-related mediators of neointimal VSMC accumulation

Agent	Action
Serotonin	Vasoconstrictor[a], VSMC mitogen
Thromboxane A_2	Vasoconstrictor, VSMC mitogen
PDGF	Vasoconstrictor, VSMC mitogen, VSMC chemotaxis
Thrombin	Vasoconstrictor[a], VSMC mitogen monocyte chemotaxis, activates MMP-1,2
Factor Xa	SMC mitogen
TGF-β	Stimulates ECM production

Note:
[a] In atherosclerotic arteries.

Experimental antithrombotic gene therapy

Compared to the large number of gene transfer approaches aimed at inhibiting VSMC proliferation, experimental gene therapy approaches for the prevention of arterial thrombosis have been limited. In part, this may reflect the greater technical challenge posed by developing thrombosis models for gene therapy. Since there is usually a delay between gene transfer and gene expression, the therapeutic test (i.e. the thrombotic challenge) requires at least one additional experiment, usually several days after the gene vector is delivered to the artery. Thrombosis is

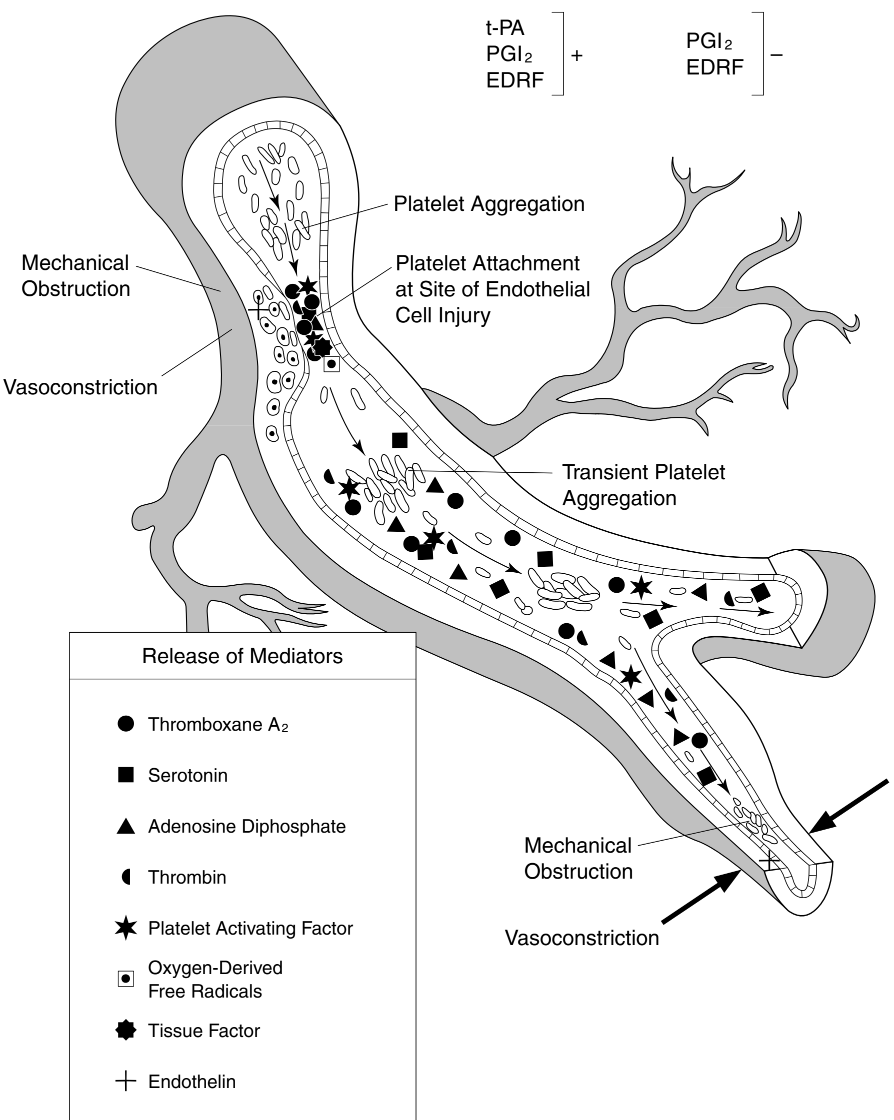


Fig. 66.1. Schematic diagram suggests probable mechanisms responsible for the conversion from chronic coronary heart disease to acute coronary artery disease syndromes. In this scheme, endothelial injury, generally at sites of atherosclerotic plaques and usually plaque ulceration or fissuring, is associated with platelet adhesion and aggregation and the release and activation of selected mediators, including thromboxane A_2, serotonin, adenosine diphosphate, platelet-activating factor, thrombin, oxygen-derived free radicals, and endothelin. Local accumulation of thromboxane A_2, serotonin, platelet-activating factor, thrombin, adenosine diphosphate, and tissue factor promotes platelet aggregation. Thromboxane A_2, serotonin, thrombin, and platelet activating factor are vasoconstrictors at sites of endothelial injury. Therefore, the conversion from chronic stable to acute unstable coronary heart disease syndromes is usually associated with endothelial injury, platelet aggregation, accumulation of platelet and other cell-derived mediators, further platelet aggregation, and vasoconstriction, with consequent dynamic narrowing of the coronary artery lumen. In addition to atherosclerotic plaque fissuring or ulceration, other reasons for endothelial injury include flow shear stress, hypertension, immune complex deposition and complement activation, infection, and mechanical injury to the endothelium as it occurs with coronary artery angioplasty and after heart transplantation. EDRF, endothelium-derived relaxing factor, PGI_2, prostaglandin I_2; t-PA, tissue-type plasminogen activator. (From Willerson JT: *Treatment of heart disease*. New York: Gower Medical, 1992; modified from Willerson JT, Golino P, Eidt JF et al. Specific platelet mediators and unstable coronary artery lesions. Experimental evidence and potential clinical implications. *Circulation* 1989; 80:198.

Cambridge Books Online © Cambridge University Press, 2009

often provoked by endothelial injury and increasing shear stress and thrombus formation is monitored in vivo by measurement of blood flow (or a surrogate such as flow velocity) or assessed after death by histomorphometry of the thrombosis-covered luminal area. Another issue, when the thrombotic test is delayed several days after the administration of the gene, is the clinical relevance of providing antithrombotic protection beginning 1 or more days after the insult. Thrombosis in patients with atherosclerotic disorders is a dynamic process, posing a lifelong risk. Chronically recurrent cycles of thrombosis, thrombolysis, thrombus organization, and rethrombosis encroach on the vascular lumen over the course of months and years and provide a myriad of stimuli for VSMC and ECM accumulation. In addition, whether involving heart, brain, or other structures, the final trigger of tissue death caused by the atherosclerotic process is formation of an occlusive thrombus. Thus, we believe that more durable and continuous antithrombotic protection may exert benefits beyond those provided by antithrombotic drug therapy during the first 24 h after the iatrogenic insult. Vectors that can express foreign genes within hours of their delivery have been described, but presently often have the trade-off of very short-lived expression[9].

Gene transfer techniques

Endothelial injury and dysfunction result in local deficiency of vasoprotective molecules, including prostacyclin (PGI_2), NO, tissue-type plasminogen activator (tPA), thrombomodulin, and tissue factor pathway inhibitor (TFPI), with an attendant risk of thrombosis. Endothelial proteins synthesizing vasoprotective molecules, such as cyclooxygenase-1 (COX-1) and endothelial nitric oxide synthase (eNOS), are membrane-bound and synthesize autacoids with short half-lives. Thus, restoration and augmentation of these autacoids by local gene transfer of crucial enzymes to sites of endothelial injury appears to be a physiologically sound strategy. In contrast to pharmacological approaches, local gene transfer techniques restrict the presence of a potentially therapeutic protein to the vascular site at risk, while extending in time the effect of a single administration of the therapeutic precursor, the DNA encoding a potentially beneficial protein. Genes transferred to the arterial wall to restore or augment the endogenous antithrombotic defence include COX-1 (catalysing PGI_2 synthesis), thrombomodulin (enhancing protein C activation), tPA (generating plasmin), and most recently TFPI, which inhibits factor Xa and the tissue factor/factor VIIa complex.

Gene transfer of thrombomodulin

Thrombomodulin, an endothelial transmembrane protein containing six tandemly repeated EGF domains, binds thrombin and its active precursors, meizothrombin and desmeizothrombin. After binding to thrombomodulin, thrombin loses its affinity for fibrinogen and, concurrently, becomes a potent activator of circulating protein C, which inactivates factors Va and VIIIa. Compared to thrombin alone, protein C activation is accelerated 20 000-fold by the endothelial thrombomodulin–thrombin complex[10]. Luminal surface expression of thrombomodulin decreases after perturbation of endothelial cells, after removal of endothelial cells (during angioplasty), and in saphenous veins after harvesting the vein[11]. Systemic infusion of recombinant thrombomodulin and of recombinant activated protein C prevents thrombosis and accelerated thrombolysis in animal models of arterial injury[12].

Shenaq's group has described a rabbit arterial stasis/minimal injury model designed to study prevention of small vessel thrombosis at sites of anastomoses[13]. In its adaptation to gene therapy studies, division of the common femoral artery was followed by microsurgical reanastomosis of the segments and adenovirally mediated gene delivery to the reanastomosed vessel. Three days later, two perpendicular sutures loops were loosely placed through the lumen distal to the anastomosis and animals were allowed to survive for an additional 3 days. At death, the thrombus area was assessed by quantitative histomorphometry. In this model of 'microvascular thrombosis', the common femoral artery was targeted by adenoviral vector injection through the inferior epigastric artery. Trauma to the endothelium was deliberately avoided. Local endothelial gene transfer of thrombomodulin in this anastomosis model reduced arterial thrombosis formation compared to viral and nonviral controls[14]. By histological analysis, delivery of the viral suspension buffer alone and of a viral control vector was associated with thrombi occluding $71 \pm 4\%$ and $87 \pm 3\%$ of the lumen, respectively. In contrast, adenoviral delivery of the thrombomodulin gene (titre of 3 $\times$109 pfu/ml) reduced the thrombus area to $29 \pm 3\%$ and significantly decreased local infiltration of granulocytes and macrophages compared to nonviral and viral control. Thrombus was exclusively assessed postmortem[14]. No information was provided about dynamic changes in thrombus formation over time or under the influence of added prothrombotic stimuli, such as systemically administered epinephrine (see below).

Cambridge Books Online © Cambridge University Press, 2009

Gene restoration of tPA

Some of the first reports of gene transfer of plasminogen activators against arterial thrombosis were made by Dicheck et al.[15]. Using recombinant retroviral vectors, these co-worker transduced cultured baboon endothelial cells ex vivo with tPA and a urokinase-type plasminogen activator variant (a-UPA). The transduced endothelial cells were instilled into exteriorized arteriovenous femoral shunts grafted with collagen. The authors were able to show a reduction in ^{111}In-labelled platelet deposition in areas covered by 'sparsely attached' endothelial cells over-expressing tPA. However, in a follow-up paper, the authors reported that the transduced endothelial cells were rapidly lost from the luminal surface. Plasmin, generated under influence of the transgene product itself (tPA, a-UPA), severely comprised the anchorage of the endothelial producer cells[16].

Gerard et al. reported in a mouse model that intravenous injection of an adenovirus encoding wild-type and a plasminogen activator inhibitor-1 (PAI-1)-resistant variant of tPA human to mice with inactivated tPA gene achieved restoration of circulating tPA[17]. Typically, systemically injected adenovirus localizes to $>$95% in the liver from which the secreted transgene product enters the blood stream[18]. In the mice injected with the viral construct, plasma levels of tPA antigen and activity were increased approximately 1000-fold above normal levels. The lysis of 1251-fibrin-labelled pulmonary emboli closely correlated with plasma tPA activity and clot lysis was obtained after injection of adenoviral constructs encoding wild-type and inhibitor-resistant tPA. Adenovirus-mediated tPA gene transfer was found to augment clot lysis as early as 4 h after infection, but expression was lost within 7 days. The authors concluded that adenovirus-mediated transfer of a tPA gene can effectively increase plasma fibrinolytic activity and either restore (in t-PA-deficient mice) or augment (in PAI-1-overexpressing mice) the thrombolytic capacity in this animal model[17].

While these mice experiments serve as a corollary to the physiological importance of circulating tPA, they do not enhance our understanding of the potential of local tPA gene therapy for the prevention of arterial thrombosis. This issue was directly addressed in recent study by Waugh et al. in the rabbit stasis-minimal injury model[19]. An adenoviral construct encoding human wild-type tPA was delivered to the minimally injured common femoral artery of New Zealand White rabbits. Three days later, thrombus formation was initiated by transection of the artery, immediate re-anastomosis and placement through the lumen of two perpendicular 8.0 silk loops distal to the anastomosis. After death on day 6 (3 days after placement of the anastomosis), the cross-sectional area of thrombotic obstruction was found to be 68 ± 8, 80 ± 6, $61 + 6$ and 19 ± 2% for rabbits treated locally with, respectively, buffer, control virus, a systemic rtPA infusion, and Ad-tPA (5×10^9 pfu/ml). Systemic hemostatic variables and plasma fibrin split products were unchanged from baseline. In this study, local expression of tPA was more effective than a systemic tPA infusion and completely spared the systemic hemostatic system[19].

Gene transfer of cyclooxygenase-1 to sites of severe arterial injury

Like tPA and NO, prostacyclin (PGI_2) is a vasoprotective molecule constitutively expressed by the healthy endothelium. PGI_2 is a potent platelet inhibitor and vasorelaxant molecule that acts on platelets on the luminal side and on VSMC underneath the endothelium. It inhibits platelet secretion and aggregation, relieves vascular contraction, suppresses VSMC proliferation, reduces lipid accumulation in mononuclear and VSMC, and is reported to block the interaction between monocytes and endothelium[20,21]. Its actions involve receptor-mediated activation of adenylyl cyclase, leading to increased intracellular cAMP levels. Attempts to prevent thrombosis by systemic infusion of prostacyclin or its stable analogues have been unsatisfactory because of unacceptable side effects.

COX-1 is considered to be the key rate-limiting step in endothelial PGI_2 synthesis[22]. Maintenance of physiological levels of PGI_2 requires continuous synthesis of this autacoid by COX-1 and other enzymes. Like its product, however, COX-1 has a relatively short half-life (about 7 min) due to autoinactivation, i.e. accelerated degradation during catalysis of PGI_2. Thus, maintenance of physiological PGI_2 levels requires continuous synthesis of COX-1 and is exquisitely vulnerable to endothelial insults[22].

In order to overcome the limitations of PGI_2 synthesis, we constructed an adenovirus encoding COX-1 (Ad-COX-1). Compared to a control vector and buffer only, gene transfer of COX-1 enhanced PGI_2 synthesis in cultured VSMC and in balloon injured porcine carotid arteries about fivefold. When porcine carotid arteries were treated for 30 min with Ad-COX-1 at a relatively high titre (6×10^{10} pfu/ml) immediately after balloon injury (5 inflations at 7 atm), cyclic flow variations (CFVs), caused by recurrent embolizing thrombi, were completely prevented for 10 days after surgery (as observed on continuous blood flow velocity recordings) and no thrombi were seen at death, suggesting that local enhancement of PGI_2 synthesis is

Cambridge Books Online © Cambridge University Press, 2009

highly effective against platelet-rich thrombosis at sites of severe arterial injury[23].

Gene restoration of tissue factor pathway inhibitor

Recently, we[24] and others[25] have begun to study the antithrombotic efficacy of gene transfer of tissue factor pathway inhibitor (TFPI). TFPI is an endogenous inhibitor of factor Xa and of tissue factor (TF), the cellular initiator of thrombin generation and blood coagulation in hemostasis and thrombosis[26]. TF-driven thrombin generation plays a pivotal role in arterial and venous thrombosis, leads to activation of enzymes involved in neointima formation[27], and is a potent VSMC mitogen[28]. Factor Xa is a VSMC mitogen as well[29]. Up-regulation of TF contributes importantly to the thrombogenicity of the atherosclerotic plaque[30]. Although systemic administration of TFPI has been studied as a new approach to the prevention of thrombosis, and restenosis[31–33], it is uncertain whether short-term administration of recombinant TFPI will achieve lasting vasoprotection, particularly at sites of increased tissue factor burden. In addition, while continuous administration of antithrombin agents, including desirudin, may promote long-term patency of injured vessel[34], hemorrhagic risks are inevitable and the systemic doses of recombinant human TFPI capable of preventing arterial thrombosis and, potentially, restenosis are substantial (100 (g/kg/min in[32]), raising further questions on the practicality of a systemic dosing approach. The need for such a high dosage has substantially tempered the enthusiasm for the development of recombinant TFPI for the treatment of arterial thrombotic disorders.

In order to study the effects of TFPI gene transfer on thrombus formation at sites of severe arterial injury, porcine carotid arteries were balloon-injured and locally treated with an adenovirus encoding human wild-type TFPI (Ad-TFPI) or a control virus. Gene transfer of TFPI was confirmed by detection of human TFPI in the conditioned medium of porcine carotid arteries kept in culture after in vivo vector delivery. When carotid flow was measured by Doppler probes 5 days after surgery, cyclic flow variations (CFVs) developed in seven of eight control pigs after constriction of the injured carotid artery by 40%, and all control treated arteries occluded after 70% constriction. In contrast, CFVs were observed in only one of eight Ad-TFPI treated pigs after 40% constriction, and only three of eight occluded after constriction by 70% ($P = 0.0027$ and $P = 0.007$, respectively). None of the five TFPI-transduced arteries open after 70% constriction developed CFVs during an incremental epinephrine infusion. A detailed analysis of coagulation variables and BT on the day of the thrombosis challenge (day 0) illustrates the potential of local arterial gene therapy to exert vasoprotection in the complete absence of systemic effects. Indeed, neither coagulation variables nor BT were significantly different between the Ad-TFPI and the control group and between baseline (day 0) and day 5. Platelet aggregation was completely conserved after local treatment with Ad-TFPI. Whether overexpression of TFPI and COX-1 at sites of increased thrombogenicity will provide lasting protection against thrombus formation and reduce the degree of neointima formation needs to be determined in future experiments.

Gene therapy to prevent fibroproliferative lesions

Local cell proliferation and migration, apoptosis, vascular remodelling, ECM deposition, and episodes of recurrent clinical and subclinical platelet deposition and thrombosis determine the magnitude of neointima formation and restenosis after percutaneous revascularization[6]. Vascular injury during PTCA/stenting stimulates proliferation and migration of vascular smooth muscle cells (VSMC) and the deposition of ECM by the 'activated' VSMC[35,36]. This role of VSMC in the restenotic process was first recognized in a rat carotid injury model. In this model, VSMC begin to proliferate within days after injury and soon migrate to the intima, helped by their production of hyaluronidase, urokinase, matrix metalloproteinases (MMP) and perhaps tPA[37,38]. There, the VSMC proliferate further and synthesize ECM and other paracrine and autocrine growth factors and cytokines, amplifying the cellular and non-cellular responses to injury. After 3–6 months, 90% of the neointimal cell population are VSMC. However, the bulk of VSMC constitutes only 20% of the neointimal mass, with most of the rest being ECM[37,39,40].

The growth of VSMC and extracellular matrix deposition are driven by a variety of growth factors, including bFGF[41], PDGF[42–44], TGF-β[45], IGF-1[46] and angiotensin II[47]. Loss of endothelial inhibitors of VSMC proliferation and inhibitors of collagen and proteoglycan synthesis, like NO, shift the balance further towards proliferation and migration of VSMC and the accumulation of ECM[48,49].

The main cellular target of antiproliferative gene therapy to date has been VSMC. The proteins encoded by the gene vector include cell cycle-regulatory proteins that inhibit entry or progression of the cell cycle ('cytostatic gene products'), or cause cell cycle exit by programmed cell death

Cambridge Books Online © Cambridge University Press, 2009

and elimination of the VSMC ('cytotoxic or apoptotic gene products'). Other transferred genes encode endothelial proteins with diverse functions, some of which affect the cell cycle machinery. Others suppress VSMC proliferation by accelerating endothelial healing of the injured vascular site, making available to the injured site the broad range of endothelial VSMC growth inhibitors. Using gene transfer techniques, the cell cycle inhibitory proteins can be overexpressed at the site of injury (e.g. adenovirus-mediated overexpression of p21 or Rb). Alternatively, the expression of cell cycle-promoting proteins can be blocked by the delivery of specific, short DNA sequences. These specific, short DNA sequences function by either decoy or antisense mechanism (see below). Both are intended to inhibit the translation of specific mRNA sequences and, thus, the synthesis of cell cycle regulatory proteins. While this latter strategy is attractive in principle, there is some concern about the specificity of the target(s) sequence inhibited by decoy and especially antisense oligodeoxynucleotides ('ODN'). On the other hand, this approach may avoid potential side effects related to the overexpression of cell cycle proteins with, as yet, unknown functions and risks.

Inhibiting cell cycle

In order to block the entry into the cell cycle and replication of the cell, a variety of gene transfer approaches are under investigation. Chang et al.[50] showed in a rat and porcine balloon injury model that adenovirus mediated transfer of a mutant gene encoding a hypophosphorylated retinoblastoma significantly reduced the intima–media ratio compared to non-treated or control-treated animals. This mutant is constitutively active and binds to and blocks E2F, a family of transcription factors that plays a major role in S-phase entry of the cell. An alternative approach uses the overexpression of the endogenous cell-cycle-dependent kinase inhibitors protein, p21[51,52], p16[52,53], and p27[54]. These proteins act upstream of Rb and function by maintaining Rb in a hypophosphorylated (inactive) state[55].

Gax is a homeobox gene normally expressed by vascular smooth cells that is down-regulated following vascular injury[56]. Overexpression by gene transfer of this gene prevents neointimal hyperplasia, but does not affect re-endothelialization and vasomotion[57]. Inhibition of cellular *ras* has also been reported to reduce neointimal thickening in a rat carotid injury model[58,59].

Promoting apoptosis

The Herpes simplex virus thymidine kinase (Hstk) is toxic when combined with ganciclovir administration. Transduced cells, expressing the thymidine kinase, phosphorylate inactive ganciclovir into the active compound, ganciclovir phosphate, which inhibits DNA polymerase (thereby blocking DNA synthesis) and results in apoptosis of the transduced cells. Using the adenovirally mediated HStk gene transfer strategy, originally developed as an approach to cancer treatment, neointimal proliferation was reduced by 50% in rat and porcine arterial injury models[60,61]. Importantly, adenovirus-mediated transfer of the HStk gene inhibited neointima formation in hyperlipidemic rabbit iliac injury model, suggesting that approach may be effective in truly atherosclerotic arteries[62]. Adenovirus-mediated gene transfer of cytosine deaminase and co-administration of 5-fluorocytosine showed similar results but may be associated with more toxicity[63].

An innovative approach is inhibition of neointima formation by transfer of proapoptotic genes. Gene transfer of the Fas ligand (activating FAS-dependent apoptosis) was shown to markedly suppress growth of cultured rat VSMC and neointima formation in a rat carotid balloon injury model at unusually low adenoviral doses (8×10^{10} pfu/ml)[64]. Coadministration of Ad-FasL with an adenoviral vector transferring an *E. coli* LacZ 'reporter' gene (encoding the enzyme, betagalactosidase, which is easily visualized by a histochemical reaction) results in markedly prolonged expression of the transferred beta-galactosidase, suggesting a role for the FasL-triggered death pathway in suppressing the immune response elicited by adenovirus-mediated gene transfer. Taking another approach, we recently reported that gene transfer of the transcription factor, E2F-1, induced S-phase entry in serum deprived human coronary VSMC, followed by rapid development of apoptosis and marked growth suppression of the VSMC. In vitro, we found that human umbilical venous endothelial cells also underwent apoptosis after transfer of E2F-1[65]. Losardo et al. reported that adenovirus-mediated gene transfer of E2F-1 protected cultured bovine endothelial cells from TNF-induced apoptosis and, at sites of balloon injury in rat carotid arteries, promoted endothelial regrowth and reduced neointima formation[66]. These apparently contradictory in vitro findings warrant clarification. Nonetheless, we have also observed marked suppression of neointima formation (in the absence of pseudoaneurysm formation) in balloon injured carotid arteries of hyperlipidemic rabbits at 28 days after the intervention[67]. Thus, gene transfer of E2F-1 may deserve further study as an approach to the prevention of neointima formation after vascular injury.

Cambridge Books Online © Cambridge University Press, 2009

Antisense oligodeoxynucleotides and decoy sequences

Expression of genes involved in cell cycle initiation and progression can be inhibited by introduction into the host nucleus of DNA sequences that contain short 'consensus' sequences, i.e. sequences that bind and inhibit several (or all) members of a family of transcription factors. An example for this strategy is the transfection into cells of double-stranded E2F decoy sequence that inhibits expression of transcription factors of the E2F family. Transfection of an E2F decoy sequence into balloon-injured rat carotid arteries markedly suppressed neointima formation[68].

Antisense oligonucleotides

Antisense oligonucleotides are short DNA sequences designed to bind and render silent specific mRNA sequences. The synthetic antisense oligonucleotides are complementary to the mRNA encoded by the gene and their mechanism of action is complex involving hybridization of mRNA and the synthetic oligonucleotide, activation of ribonucleases H and L and elimination of the mRNA[69]. Oligonucleotides against transcriptional or regulatory proteins have been designed, including those binding to the mRNA of c-myb, Cdk2, NFkB and others. Local administration of antisense oligonucleotides directed against cell cycle-promoting factors, including proliferating cell nuclear antigen (PCNA, an essential cofactor for the principle DNA polymerase, pol-δ), the cell cycle-dependent kinase 2 (Cdk2), and other cell cycle promoting factors were reported to inhibit the proliferation of VSMC in vitro and formation of intimal hyperplasia in vivo[70–80]. In a rabbit vein graft model, a combined administration of antisense ODN against PCNA and Cdk2 inhibited neointimal formation and atherosclerosis hyperplasia[81]. This latter strategy has been tested in the first human trial of gene therapy against vein graft deterioration (see below). Problems with the transfection of antisense ODN include their stability within the cell, their specificity and mechanism of action, and possible effects related to the liposomal transfection method itself[69,82]. A somewhat related approach (aiming at silencing the mRNA of target proteins) is delivery of ribozymes (especially of the hammerhead type) to the arterial wall. This approach has been studied in the vasculature only preliminarily[83–85].

Gene therapy inhibiting migration of VSMC

Another approach to the prevention of neointima formation involves the blockade of signals promoting VSMC migration. Like hyaluronidase, plasmin facilitates migration of VSMC through the ECM. Plasminogen activator-inhibitor-1 (PAI-1) is the endogenous inhibitor of the plasminogen activators, urokinase (UPA) and tPA. Carmeliet et al. demonstrated that systemic adenovirus mediated gene transfer of PAI-1 to mice with inactivated PAI-1 gene reduced neointima formation in injured carotid arteries from a cross-sectional luminal reduction ('restenosis') of 35% in the control vector treated animals to 5% in the Ad-PAI-1-treated animals[86]. On the other hand, the same vector (Ad-PAI-1) did not inhibit neointima formation after coronary balloon injury in normal pigs[87].

An essential component in the pathogenesis of the neointima in injured arteries and vein grafts is migration of cells, especially VSMC, through the ECM towards the vascular lumen. This process is markedly facilitated by the enhanced synthesis matrix metalloproteinases (gelatinases, MMP), especially MMP-2 and MMP-9[88–93]. Several groups have reported that gene transfer of TIMP-1 and -2 to injured rat balloon arteries and saphenous veins kept in organ culture markedly inhibited neointima formation[89,94,95].

Promoting nitric oxide synthase (NOS) and vascular endothelial growth factor (VEGF)

Nitric oxide (NO) is a regulator of vasomotion, platelet aggregation, VSMC proliferation and migration, adhesion of monocytes, and promotes the synthesis of vascular endothelial growth factor (VEGF). At high concentrations (such as those produced by the inducible form of NOS, iNOS), NO impairs the function of many proteins by nitration of tyrosine residues and it induces p53 and apoptosis in VSMC and other cells[96,97]. NO (like PGI_2) is reduced after vascular injury and can be restored by local gene transfer of eNOS. eNOS produces NO in a non-toxic, picomolar concentration, whereas iNOS produces NO in the nanomolar range. Gene transfer of eNOS has been conclusively shown to reduce neointimal formation in balloon-injured and stented rat carotid arteries and in balloon-injured porcine coronary arteries[98–100]. Endovascular and adventitial delivery of the NOS genes has been shown to restore vasorelaxation in carotid arteries of cholesterol-fed and Watanabe rabbits (the rabbit model of human familial hypercholesterolemia) and cerebral arteries in rats[101–103]. The potential of eNOS gene therapy (which was more

Cambridge Books Online © Cambridge University Press, 2009

efficacious than ganciclovir) plus gene transfer of HStk in a head-to-head comparison of gene therapy for the prevention of neointima formation in balloon injured porcine coronary arteries[87], is substantial and clinical trials are imminent. Gene transfer of *iNOS* was found to prevent restenosis after balloon injury, transplant arteriopathy, and may find other potential application[104,105].

Gene transfer of VEGF and more recently, hepatic growth factor (hGF, scatter factor[106]) has been under intense investigation. VEGF not only promotes growth of new microvasculature, but also accelerates re-endothelialization of injured, denuded muscular and conductive arteries, thereby reducing restenosis[107] and, potentially, thrombosis (a hypothesis yet to be tested in appropriate animal experiments).

Gene therapy and vein grafts

Veins are the most frequently used conduits to bypass stenotic arteries in the coronary or peripheral circulation.

With approximately 400 000 coronary bypass procedures (CABG) performed annually, CABG is the most commonly performed major operation in the USA. Although arterial conduits, such as the internal mammary artery, have a longer patency rate compared to grafted veins, saphenous vein grafts represent over 70% of grafts because of their plentiful availability, easy accessibility, and the simplicity of the harvesting procedure. Due to the higher pressures and pulsatility of the arterial circulation, and factors related to the surgical procedure (focal endothelial loss, ischemia of the graft), the grafted vein undergoes a characteristic sequence of adaptive changes after its insertion into the arterial circulation. In the presence of often persistent coronary risk factors, these 'adaptive' responses set the stage for the early and late graft failure. The suboptimal patency of vein grafts begins with a thrombotic occlusion rate of 8–18% within the first month. One year after surgery, 15–30% of vein grafts are stenosed and 50% of vein grafts are occluded at 10 years[108–110]. Repeated CABG procedures are associated with increased surgical risk and higher occlusion rates compared to the initial graft and now account for 10–20% of CABG surgery performed in the USA. Although relatively small trials have shown that aggressive lipid-lowering strategy, smoking cessation and control of hyperglycemia may have beneficial influences on late graft patency[108,111,112], no pharmacological approach has reduced the incidence of vein graft failure to the single digit range.

Implications for gene delivery

Some special considerations apply to transduction of vein grafts. Native veins are thin and weak. A single layer of endothelial cells is separated from the media by a rudimentary internal elastic membrane. The media consists of several layers of VSMC, which in general are circular oriented. However, longitudinally oriented VSMC are often found in the innermost layers. The adventitial tissue is composed of collagen, scattered VSMC, many elastic fibres and vasa vasorum. In contrast, the artery is constructed much more compactly. The thick media consists of VSMC and elastic fibres that enable the artery to resist high blood pressure and other forms of stress.

Three major mechanisms involved in the development of early and late vein graft failure are: thrombosis, fibrointimal hyperplasia, and graft atherosclerosis[108].

Recurrent thrombosis plays a role in early (within 1 month) graft failure and the chronic progression of vein graft disease and is, in general, the immediate cause of vein graft-related myocardial infarction and ischemic death. Tissue factor has been shown, at least experimentally, to accumulate early in vein grafts[113], and may play a role in all stages of vein graft deterioration. Endothelial cell loss and vessel injury also impair the availability of cell-based antithrombotic molecules, including thrombomodulin, PGI_2 and NO. Recurrent mural platelet thrombus formation contributes to narrowing and, often occlusion of the graft at all stages[108].

Beyond the first month after surgery, intimal 'hyperplasia' becomes a prominent determinant of vein graft deterioration.

After the first year, the vein graft undergoes atherosclerotic changes very similar to those observed in native arteries, although the atherosclerotic process in vein grafts appears to be more diffuse and concentric. Atherosclerotic plaques developing in vein grafts seem to be weaker and thinner than in native arteries. Therefore, the risk of plaque rupture might be greater in vein grafts compared to arteries of comparable size and, in concert with the accumulation of TF in the vein grafts, may play a role in the higher thrombus burden observed in vein grafts compared to arterial conduits.

Gene therapy to prevent vein graft deterioration

Treatment of vein grafts with gene vectors represents an opportunity to incubate the vessel for relatively long time periods (20–40 min) with the viral solution before

Cambridge Books Online © Cambridge University Press, 2009

interpositioning the vein into the artery[114]. By limiting gene transfer to an ex vivo procedure, the risk of systemic virus distribution is probably very limited. However, the optimal vector for vein graft transduction is uncertain. Adenoviral vectors, currently the most efficient vectors for direct vascular gene transfer, circumvent the need for autologous cell preparation. However, at concentrations of 5×10^9 pfu/ml, they elicit a marked inflammatory response, which may lead to cell death, tissue destruction, and accelerated neointima formation and in general limits foreign gene expression to 7–10 days. Thus, Channon et al. detected Ad-LacZ transduced endothelial cells and infrequently transduced medial VSMC in rabbit jugular vein grafts 3 days after intrasurgical gene delivery, but reported that transgene expression was markedly decreased by day 7[115]. Consequently, for the use of adenoviral vectors, the therapeutic 'window of opportunity' may involve a narrow dose range. In contrast 'recombinant adeno-associated virus-based vectors' (rAAV) are small (20 nm), appear to transduce all layers of the artery even in the absence of vascular injury[116,117], elicit little, if any, inflammation, and can drive transgene expression for more than 18 months[118,119]. Like adenovirus-mediated gene transfer, rAAV-based gene transfer does not require dividing target cells. However, gene expression after rAAV is relatively slow in onset and no reports of rAAV-mediated LacZ expression within the first 6 days after vascular gene transfer are available.

Chen et al. were the first to report on adenovirus-mediated gene expression in a (porcine) vein graft in vivo[114]. Gene transfer involves in general ex vivo incubation of the rabbit jugular vein with the gene vector, division of the carotid artery, and insertion of the transduced vein in reversed fashion into the divided carotid artery with creation of end-to-end or end-to-side anastomoses to restore carotid flow. After surgery, rabbits are generally fed with 0.75–1% cholesterol-enriched food to allow the development of 'atherosclerotic' (lipid-infiltrated) neointimal lesion in the grafted jugular veins.

Mann et al. pioneered the use of antisense ODN targeting genes encoding cell-cycle proteins in the rabbit jugular vein graft model. Cdk2 and PCNA expression in grafted vein was found to be increased, respectively, ten and fivefold after surgery. Using liposomes complexed with proteins from the hemagglutinating virus of Japan (HVJ), a single ex vivo treatment of the grafts with the antisense ODN reduced expression of Cdk2 and PCNA by 90% compared to control treated animals and markedly suppressed formation of lipid-rich ('atherosclerotic') neointimal lesion formation[81]. Other approaches shown to be effective in inhibiting atherosclerosis/neointima formation in rabbit carotid grafts were ex vivo adenovirus-mediated gene transfer of a specific inhibitor of G-β,γ[120] and a hypophosphorylated, constitutively active form of the retinoblastoma protein[121].

Conclusion

We predict that improved gene transfer vectors and simplified vector delivery systems should allow the clinician in the not too distant future to use gene therapy to prevent thrombosis and restenosis after (and potentially before) vascular injury.

References

1 Heijer P den, Dijk RB van, Hillege HL, Pentinga ML, Serruys PW, Lie KI. Serial angioscopic and angiographic observations during the first hour after successful coronary angioplasty: a preamble to a multicenter trial addressing angioscopic markers of restenosis. *Am Heart J* 1994; 128: 656–663.
2 Leimgruber PP, Roubin GS, Hollman J et al. Restenosis after successful coronary angioplasty in patients with single vessel disease. *Circulation* 1986; 73: 710–717.
3 Rupprecht JH, Brennecke R, Kottmeyer M et al. Short- and long-term outcome after PTCA in patients with stable and unstable angina. *Eur Heart J* 1990; 11: 964–973.
4 Fuster V, Falk E, Fallon JT, Badimon L, Chesebro JH, Badimon JJ. The three processes leading to post PTCA restenosis: dependence on the lesion substrate. *Thromb Haemost* 1995; 74: 552–559.
5 Willerson JT, Yao SK, McNatt J et al. Frequency and severity of cyclic flow alternations and platelet aggregation predict the severity of neointimal proliferation following experimental coronary stenosis and endothelial injury. *Proc Natl Acad Sci USA* 1991; 88: 10624–10628.
6 Chen L, Chester MR, Redwood S, Huang J, Leatham E, Kaski JC. Angiographic stenosis progression and coronary events in patients with 'stabilized unstable angina'. *Circulation* 1995; 91: 2319–2324.
7 Topol EJ, Califf RM, Weisman HF et al. Randomised trial of coronary intervention with antibody against IIb/IIIa integrin for reduction of clinical restenosis: results at six months. The EPIC Investigators. *Lancet* 1994; 343: 881–886.
8 Lincoff AM, Tcheng JE, Califf RM et al. Sustained suppression of ischemic complications of coronary intervention by platelet GP IIb/IIIa blockade with abciximab: one-year outcome in the EPILOG trial. Evaluation in PTCA to improve long-term outcome with abciximab GP IIb/IIIa blockade. *Circulation* 1999; 99: 1951–1958.
9 Agapov EV, Frolov I., Lindenbach BD, Pragai BM, Schlesinger S, Rice CM. Noncytopathic Sindbis virus RNA vectors for heterologous gene expression. *Proc Natl Acad Sci USA* 1998; 95: 12989–12994.

Cambridge Books Online © Cambridge University Press, 2009

10 Sadler JE. Thrombomodulin structure and function. *Thromb Haemost* 1997; 78: 392–395.
11 Motwani JG, Topol EJ. Aortocoronary saphenous vein graft disease: pathogenesis, predisposition, and prevention. *Circulation* 1998; 97: 916–931.
12 Grinnell BW, Yan SB. Novel antithrombotics based on modulation of the protein C pathway. *Coron Artery Dis* 1998; 9: 89–97.
13 Shenaq SM, Kattash MM, Weinfeld AB et al. Local gene delivery: arterial thrombosis model for endothelial cell-targeted thrombolytic gene therapy research. *J Reconstr Microsurg* 1999; 15: 73–79.
14 Waugh JM Yuksel E, Kuo MD et al. Local overexpression of thrombomodulin for in vivo prevention of arterial thrombosis in a rabbit model. *Circ Res* 1999; 84: 84–92.
15 Dichek DA, Anderson J, Kelly AB, Hanson SR, Harker LA. Enhanced in vivo antithrombotic effects of endothelial cells expressing recombinant plasminogen activators transduced with retroviral vectors. *Circulation* 1996; 93: 301–309.
16 Dunn PF, Newman KD, Jones M et al. Seeding of vascular grafts with genetically modified endothelial cells. Secretion of recombinant TPA results in decreased seeded cell retention in vitro and in vivo. *Circulation* 1996; 93: 1439–1446.
17 Carmeliet P, Stassen JM, Van Vlaenderen I, Meidell RS, Collen D, Gerard RD. Adenovirus-mediated transfer of tissue-type plasminogen-activator augments thrombolysis in tissue-type plasminogen activator-deficient and plasminogen activator inhibitor-1-overexpressing mice. *Blood* 1997; 90: 1527–1530.
18 Herz J, Gerard RD. Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice. *Proc Natl Acad Sci USA* 1993; 90: 2812–2816.
19 Waugh JM, Kattash M, Li J et al. Gene therapy to promote thromboresistance: local overexpression of tissue plasminogen activator to prevent arterial thrombosis in an in vivo rabbit model. *Proc Natl Acad Sci USA* 1999; 96: 1065–1070.
20 Vane JR. Prostaglandins and the cardiovascular system. *Br Heart J* 1983; 49: 405–409.
21 Pomerantz K, Hajjar D. Eicosanoids in regulation of arterial smooth muscle cell phenotype, proliferative capacity, and cholesterol metabolism. *Arteriosclerosis* 1989; 9: 413–429.
22 Wu KK, Kulmacz RJ, Wang L-H, Loose-Mitchell DS, Tsai A-L. *Molecular Biology of Prostacyclin Biosynthesis*. G Rubanyi and J.R. Vane, editors. Amsterdam: Elsevier Science Publishers. B.V., 1992: 11–23.
23 Zoldhelyi P, McNatt J, Xu X-M et al. Prevention of arterial thrombosis by adenovirus-mediated transfer of cyclooxygenase gene. *Circulation* 1996; 93: 10–17.
24 Zoldhelyi P, McNatt J, Shelat HS, Yamamoto Y, Chen Z-Q, Willerson JT. Thromboresistance of balloon-injured porcine carotid arteries after local gene transfer of human tissue factor pathway inhibitor. *Circulation* 2000; 101: 289–295.
25 Nishida T, Ueno H, Atsuchi N et al. Adenovirus-mediated local expression of human tissue factor pathway inhibitor eliminates shear stress-induced recurrent thrombosis in the injured carotid artery of the rabbit. *Circ Res* 1999; 84: 1446–1452.
26 Rappaport SI, Rao LVM. Initiation and regulation of tissue factor-dependent blood coagulation. *Arterioscl Thromb* 1992; 12: 1111–1121.
27 Galis ZS, Kranzhofer R, Fenton JW II, Libby P. Thrombin promotes activation of matrix metalloproteinase-2 produced by cultured vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 1997; 483–489.
28 McNamara CA, Sarembock IJ, Gimple LW, Fenton JW II, Coughlin SR, Owens GK. Thrombin stimulates proliferation of cultured rat aortic smooth muscle cells by a proteolytically activated receptor. *J Clin Invest* 1993; 91: 94–98.
29 Gasic GP, Arenas CP, Gasic TB, Gasic GJ. Coagulation factors X, Xa, and protein S as potent mitogens of cultured aortic smooth muscle cells. *Proc Natl Acad Sci USA* 1992; 89: 2317–2320.
30 Toschi V, Gallo R, Lettino M et al. Tissue factor predicts the thrombogenicity of human atherosclerotic plaque components. *Circulation* 1997; 95: 594–599.
31 Haskel EJ, Torr SR, Day KC, Palmier MO, Wun TC, Sobel BE, Abendschein DR. Prevention of arterial reocclusion after thrombolysis with recombinant lipoprotein-associated coagulation inhibitor. *Circulation* 1991; 84: 821–827.
32 Oltrona L, Speidel CM, Recchia D, Wickline SA, Eisenberg PR, Abendschein DR. Inhibition of tissue factor-mediated coagulation markedly attenuates stenosis after balloon-induced arterial injury in minipigs. *Circulation* 1997; 96: 646–652.
33 Jang Y, Guzman LA, Lincoff M, Topol EJ. Influence of blockade at specific levels of the coagulation cascade on restenosis in a rabbit atherosclerotic femoral artery injury model. *Circulation* 1995; 92: 3041–3050.
34 Gallo R, Padurean A, Toschi V et al. Prolonged thrombin inhibition reduces restenosis after balloon angioplasty in porcine coronary arteries. *Circulation* 1998; 97: 581–588.
35 Schwartz RS, Edwards WD, Huber KC et al. Coronary restenosis: prospects for solution and new perspectives from a porcine model. *Mayo Clin Proc* 1993; 68: 54–62.
36 Gibbons GH, Dzau VJ. The emerging concept of vascular remodelling. *N Engl J Med* 1994; 330: 1431–1438.
37 Clowes AW, Reidy MA, Clowes MM. Mechanisms of stenosis after arterial injury. *Lab Invest* 1983; 49: 208–215.
38 Kenagy RD, Vergel S, Mattsson E, Benedeck M, Reidy MA, Clowes AW. The role of plasminogen, plasminogen activators, and matrix-metalloproteinases in primate arterial smooth muscle cell migration. *Arterioscler Thromb Biol* 1996; 16: 1373–1382.
39 Clowes AW, Schwartz SM. Significance of quiescent smooth muscle migration in the injured rat carotid artery. *Circ Res* 1985; 56: 139–145.
40 Clowes AW, Reidy MA, Clowes MM. Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. *Lab Invest* 1983; 49: 327–333.
41 Reidy MA, Fingerle J, Lindner V. Factors controlling the

Cambridge Books Online © Cambridge University Press, 2009

development of arterial lesions after injury. *Circulation* 1992; 86: 11143–11146.

42 Ferns GA, Raines EW, Sprugel KH, Motani AS, Reidy MA, Ross R. Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. *Science* 1991; 253: 1129–1132.

43 Jawien A, Bowen-Pope DF, Lindner V, Schwartz SM, Clowes AW. Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. *J Clin Invest* 1992; 89: 507–511.

44 Nabel EG, Yang Z, Liptay S et al. Recombinant platelet-derived growth factor B gene expression in porcine arteries induce intimal hyperplasia in vivo. *J Clin Invest* 1993; 91: 1822–1829.

45 Nabel EG, Shum L, Pompili VJ et al. Direct transfer of transforming growth factor beta 1 gene into arteries stimulates fibrocellular hyperplasia. *Proc Natl Acad Sci USA* 1993; 90: 10759–10763.

46 Grant MB,Wargovich TJ, Ellis EA, Caballero S, Mansour M, Pepine CJ. Localization of insulin-like growth factor I and inhibition of coronary smooth muscle cell growth by somatostatin analogues in human coronary smooth muscle cells. A potential treatment for restenosis? *Circulation* 1994; 89: 1511–1517.

47 Dzau VJ, Gibbons GH. Endothelium and growth factors in vascular remodelling of hypertension. *Hypertension* 1991; 18 (Suppl. III): 115–121.

48 Garg UC, Hassid A. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J Clin Invest* 1989; 83: 1774–1777.

49 Myers PR, Tanner MA. Vascular endothelial cell regulation of extracellular matrix collagen: role of nitric oxide. *Arterioscler Thromb Biol* 1998; 18: 717–722.

50 Chang MW, Barr E, Seltzer J et al. Cytostatic gene therapy for vascular proliferative disorders with a constitutively active form of the retinoblastoma gene product. *Science* 1995; 267: 518–522.

51 Chang MW, Barr E, Lu MM, Barton K, Leiden JM. Adenovirus-mediated overexpression of the cyclin/cyclin-dependent kinase inhibitor, p21 inhibits vascular smooth muscle cell proliferation and neointima formation in the rat carotid artery model of balloon angioplasty. *J Clin Invest* 1995; 96: 2260–2268.

52 Yang ZY, Simari RD, Perkins ND, San H, Gordon D, Nabel GJ, Nabel EG. Role of the p21 cyclin-dependent kinase inhibitor in limiting intimal cell proliferation in response to arterial injury. *Proc Natl Acad Sci USA* 1996; 93: 7905–7910.

53 Schreiber M, Muller WJ, Singh G, Graham FL. Comparison of the effectiveness of adenovirus vectors expressing cyclin kinase inhibitors p16INK4A, p18INK4C, p19INK4D, p21(WAF1/CIP1) and p27KIP1 in inducing cell cycle arrest, apoptosis and inhibition of tumorigenicity. *Oncogene* 1999; 18: 1663–1676.

54 Chen D, Krasinski K, Sylvester A, Chen J, Nisen PD, Andres V. Downregulation of cyclin-dependent kinase 2 activity and cyclin A promoter activity in vascular smooth muscle cells by p27(KIP1), an inhibitor of neointima formation in the rat carotid artery. *J Clin Invest* 1997; 99: 2334–2341.

55 Aprelikova O, Xiong Y, Liu ET. Both p16 and p21 families of cyclin-dependent kinase (CDK) inhibitors lock the phosphorylation of cyclin-dependent kinases by the CDK-activating kinase. *J Biol Chem* 1995; 270: 18195–18197.

56 Weir L, Chen D, Pastore C, Isner JM, Walsh K. Expression of gax, a growth arrest homeobox gene, is rapidly down-regulated in the rat carotid artery during the proliferative response to balloon injury. *J Biol Chem* 1995; 270: 5457–5461.

57 Maillard L, Van Belle E, Smith RC et al. Percutaneous delivery of the gax gene inhibits vessel stenosis in a rabbit model of balloon angioplasty. *Cardiovasc Res* 1997; 35: 536–546.

58 Indolfi C, Avvedimento EV, Rapcciuolo A et al. Inhibition of cellular ras prevents smooth muscle cell proliferation after vascular injury in vivo. *Nat Med* 1995; 1: 541–545.

59 Ueno H, Yamamoto H, Ito S, Li JJ, Takeshita A. Adenovirus-mediated transfer of a dominant-negative H-ras suppresses neointimal formation in balloon-injured arteries in vivo. *Arterioscler Thromb Vasc Biol* 1997; 17: 898–904.

60 Guzman RJ, Hirschowitz EA, Brody SL, Crystal RG, Epstein SE, Finkel T. In vivo suppression of injury-induced vascular smooth muscle cell accumulation using adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene. *Proc Natl Acad Sci USA* 1994; 91: 10732–10736.

61 Ohno T, Gordon D, San H et al. Gene therapy for vascular smooth muscle cell proliferation after arterial injury. *Science* 1994; 265: 781–784.

62 Simari RD, San H, Rekhter M, Ohno T, Gordon D, Nabel GJ, Nabel EG. Regulation of cellular proliferation and intimal formation following balloon injury in atherosclerotic rabbit arteries. *J Clin Invest* 1996; 98: 225–235.

63 Harrell RL, Rajanayagam S, Doanes AM et al. Inhibition of vascular smooth muscle cell proliferation and neointimal accumulation by adenovirus-mediated gene transfer of cytosine deaminase. *Circulation* 1997; 96: 621–627.

64 Sata M, Perlman H, Muruve DA et al. Fas ligand gene transfer to the vessel wall inhibits neointima formation and overrides the adenovirus-mediated T Cell response. *Proc Natl Acad Sci USA* 1998; 95: 1213–1217.

65 Chou MF, Shelat HS, Liu TJ, Willerson JT, Zoldhelyi P. Induction of growth suppression of human vascular smooth muscle cells by gene transfer of the transcription factor, E2F-1 (Abstract). *Circulation* 1998; 98 (Suppl I): I-598.

66 Spyridopoulos I, Principe N, Krasinski KL et al. Restoration of E2F expression rescues vascular endothelial cells from tumor necrosis factor-alpha induced apoptosis. *Circulation* 1998; 98: 2883–2890.

67 Z-Q Chen, Shelat HS, Chou MF et al. Gene transfer of E2F-1 induces apoptosis in vascular smooth muscle cells and prevents atherosclerotic lesion formation after balloon injury in hypercholesterolemic rabbits (Abstract). *Circulation* 1999 (in press).

68 Morishita R, Gibbons GH, Horiuchi M et al. A gene therapy strategy using a transcription factor decoy of the E2F binding

Cambridge Books Online © Cambridge University Press, 2009

site inhibits smooth muscle proliferation in vivo. *Proc Natl Acad Sci USA* 1995; 92: 5855–5859.

69 Agrawal S, Zhao Q. Antisense therapeutics. *Curr Opin Chem Biol* 1998; 2: 519–528.

70 Morishita R, Gibbons GH, Ellison KE et al. Single intraluminal delivery of antisense cdc2 kinase and proliferating-cell nuclear antigen oligonucleotides results in chronic inhibition of neointimal hyperplasia. *Proc Natl Acad Sci USA* 1993; 90: 8474–8478.

71 Morishita R, Gibbons GH, Ellison KE et al. Intimal hyperplasia after vascular injury is inhibited by antisense Cdk 2 kinase oligonucleotides. *J Clin Invest* 1994; 93: 1458–1464.

72 Morishita R, Gibbons GH, Kaneda Y, Ogihara T, Dzau VJ. Pharmacokinetics of antisense oligodeoxyribonucleotides (cyclin B1 and Cdc2 kinase) in the vessel wall in vivo: enhanced therapeutic utility for restenosis by HVJ-liposome delivery. *Gene* 1994; 149: 13–19.

73 Abe J, Zhou W, Taguchi J et al. Suppression of neointimal smooth muscle cell accumulation in vivo by antisense cdc2 and Cdk2 oligonucleotides in rat carotid artery. *Biochem Biophys Res Commun* 1994; 198: 16–24.

74 Degols G, Leonetti JP, Mechti N, Lebleu B. Antiproliferative effects of antisense oligonucleotides directed to the RNA of c-myc oncogene. *Nucl Acids Res* 1991; 19: 945–948.

75 Shi Y, Hutchinson HG, Hall DJ, Zalewski A. Downregulation of c-myc expression by antisense oligonucleotides inhibits proliferation of human smooth muscle cells. *Circulation* 1993; 88: 1190–1195.

76 Shi Y, Fard A, Galeo A et al. Transcatheter delivery of c-myc antisense oligomers reduces neointimal formation in a porcine model of coronary artery balloon injury. *Circulation* 1994; 90: 944–951.

77 Bennett MR, Anglin S, McEwan JR, Jagoe R, Newby AC, Evan GI. Inhibition of vascular smooth muscle cell proliferation in vitro and in vivo by c-myc antisense oligonucleotides. *J Clin Invest* 1994; 93: 820–828.

78 Simons M, Edelman ER, DeKeyser JL, Langer R, Rosenberg RD. Antisense c-myb oligonucleotides inhibit intimal arterial smooth muscle cell accumulation in vivo. *Nature* 1992; 359: 67–70.

79 Simons M, Edelman ER, Rosenberg RD. Antisense proliferating cell nuclear antigen oligonucleotides inhibit intimal hyperplasia in a rat carotid artery injury model. *J Clin Invest* 1993; 93: 2351–2356.

80 Autieri MV, Yue TL, Ferstein GZ, Ohlstein E. Antisense oligonucleotides to the p65 subunit of NF-B inhibit human vascular smooth muscle cell adherence and proliferation and prevent neointima formation in rat carotid arteries. *Biochem Biophys Res Commun* 1995; 213: 827–836.

81 Mann MJ, Gibbons GH, Kernoff RS et al. Genetic engineering of vein grafts resistant to atherosclerosis. *Proc Natl Acad Sci USA* 1995; 92: 4502–4506.

82 Scott-Burden T, Engler DA, Tock CL, Schwarz JJ, Casscells SW. Liposomal induction of NO synthase expression in cultured vascular smooth muscle cells. *Biochem Biophys Res Commun* 1997; 231: 780–783.

83 Frimerman A, Welch PK, Jin X et al. Chimeric DNA-RNA hammerhead ribozyme to proliferating cell nuclear antigen reduces stent-induced stenosis in a porcine coronary model. *Circulation* 1999; 99: 697–703.

84 Jarvis TC, Wincott FE, Alby LJ et al. Optimizing the cell efficacy of synthetic ribozymes. Site selectionband chemical modifications of ribozymes targeting the proto-oncogene c-myb. *J Biol Chem* 1996; 271: 29107–29112.

85 Jarvis TC, Alby LJ, Beaudry AA et al. Inhibition of vascular smooth muscle cell proliferation by ribozymes that cleave c-myb mRNA. *RNA* 1996; 2: 419–428.

86 Carmeliet P, Moons L, Lijnen R et al. Inhibitory role of plasminogen activator inhibitor-1 in arterial wound healing and neointima formation: a gene targeting and gene transfer study in mice. *Circulation* 1997; 96: 3180–3191.

87 Varenne O, Sinnaeve P, Gillijns H et al. Comparison of recombinant adenovirus vectors expressing antimigratory and antiproliferative proteins in a porcine coronary restenosis model (abstract). *J Am Coll Cardiol* 1998; 31 (Suppl A): 356A.

88 Zempo N, Kenagy RD, Au YP et al. Matrix metalloproteinases of vascular wall cells are increased in balloon-injured rat carotid artery. *J Vasc Surg* 1994; 20: 209–217.

89 Forough R, Koyama N, Hasenstab D et al. Overexpression of tissue inhibitor of matrix metalloproteinase-1 inhibits vascular smooth muscle cell functions in vitro and in vivo. *Circ Res* 1996; 79: 812–820.

90 Fabunmi RP, Baker AH, Murray EJ, Booth RF, Newby AC. Divergent regulation by growth factors and cytokines of 95 kDa and 72 kDa gelatinases and tissue inhibitors of metalloproteinases-1, -2, and -3 in rabbit aortic smooth muscle cells. *Biochem J* 1996; 315: 335–342.

91 George SJ, Zaltsman AB, Newby AC. Surgical preparative injury and neointima formation increase MMP-9 expression and MMP2 activation in human saphenous vein. *Cardiovasc Res* 1997; 33: 447–459.

92 Southgate KM, Mehta D, Izzat MB, Newby AC, Angelini GD. Increased secretion of basement membrane-degrading metalloproteinases in pig saphenous vein into carotid artery interposition grafts. *Arterioscler Thromb Vasc Biol* 1999; 19: 1640–1649.

93 Kranzhofer A, Baker AH, George SJ, Newby AC. Expression of tissue inhibitor of metalloproteinase-1, -2, and –3 during neointima formation in organ cultures of human saphenous vein. *Arterioscler Thromb Vasc Biol* 1999; 19: 255–265.

94 George SJ, Baker AH, Angelini GD, Newby AC. Gene transfer of tissue inhibitor of metalloproteinases-2 inhibits metalloproteinase activity and neointima formation in human saphenous veins. *Gene Ther* 1998; 5: 1552–1560.

95 George SJ, Johnson JL, Angelini GD, Newby AC, Baker AH. Adenovirus-mediated gene transfer of the human TIMP-1 gene inhibits smooth muscle cell migration and neointimal formation in human saphenous vein. *Hum Gene Ther* 1998; 9: 867–877.

96 Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite; the good, the bad, and ugly. *Am J Physiol* 1996; 271: C1424–C1437.

Cambridge Books Online © Cambridge University Press, 2009

97 Forrester K, Ambs S, Lupold SE et al. Nitric oxide-induced p53 accumulation and regulation of inducible nitric oxide synthase expression by wild-type p53. *Proc Natl Acad Sci USA* 1996; 93: 2442–2447.
98 Von der Leyen HE, Gibbons GH, Morishita R et al. Gene therapy inhibiting neointimal vascular lesion: in vivo transfer of endothelial cell nitric oxide synthase gene. *Proc Natl Acad Sci USA* 1995; 92: 1137–1141.
99 Janssens S, Flaherty D, Nong Z et al. Human endothelial nitric oxide synthase gene transfer inhibits vascular smooth muscle cell proliferation and neointima formation after balloon injury in rats. *Circulation* 1998; 97: 1274–1281.
100 Varenne O, Pislaru S, Gillijns H et al. Local adenovirus-mediated transfer of human endothelial nitric oxide synthase reduces luminal narrowing after coronary angioplasty in pigs. *Circulation* 1998; 98: 919–926.
101 Channon KM, Qian H, Neplioueva V et al. *In vivo* gene transfer of nitric oxide synthase enhances vasomotor function in carotid arteries from normal and cholesterol-fed rabbits. *Circulation* 1998; 98: 1905–1911.
102 Kullo IJ, Mozes G, Schwartz RS et al. Adventitial gene transfer of recombinant endothelial nitric oxide synthase to rabbit carotid arteries alters vascular reactivity. *Circulation* 1997; 96: 2254–2261.
103 Chen AFY, Jiang SW, Crotty TB et al. Effects of in vivo adventitial expression of recombinant endothelial nitric oxide synthase gene in cerebral arteries. *Proc Natl Acad Sci USA* 1997; 94: 12568–12573.
104 Shears LL II, Kibbe MR, Murdock AD et al. Efficient inhibition of intimal hyperplasia by adenovirus-mediated inducible nitric oxide synthase gene transfer to rats and pigs in vivo. *J Am Coll Surg* 1998; 187: 295–306.
105 Shears LL, Kawaharada N, Tzeng E et al. Inducible nitric oxide synthase suppresses the development of allograft arteriosclerosis. *J Clin Invest* 1997; 100: 2035–2042.
106 Morishita R, Nakamura S, Hayashi S et al. Therapeutic angiogenesis induced by human recombinant hepatocyte growth factor in rabbit hind limb ischemia model as cytokine supplement therapy. *Hypertension* 1999; 33: 1379–1384.
107 Asahara T, Bauters C, Pastore C et al. Local delivery of vascular endothelial growth factor accelerates reendothelialization and attenuates intimal hyperplasia in balloon-injured rat carotid artery. *Circulation* 1995; 91: 2793–2801.
108 Fuster V, Vorchheimer DA. Prevention of atherosclerosis in coronary artery bypass grafts. *N Engl J Med* 1997; 336: 212–213.
109 Paz MA, Lupon J, Bosch X, Pomar JL, Sanz G and the GESICA Study Group. Predictors of early saphenous vein aortocoronary bypass graft occlusion. *Ann Thorac Surg* 1993; 56: 1101–1106.
110 Fitzgibbon GM, Kafka HP, Leach AJ, Keon WJ, Hooper GD, Burton JR. Coronary bypass graft fate and patient outcome: Angiographic follow-up of 5065 grafts related to survival and reoperation in 1388 patients during 25 years. *J Am Coll Cardiol* 1996; 28: 616–626.
111 Post Coronary Artery Graft Trial Investigators. The effect of aggressive lowering of low-density lipoprotein cholesterol levels and low-dose anticoagulation on obstructive changes in saphenous-vein coronary-artery bypass grafts. *N Engl J Med* 1997; 336: 153–162 (Published erratum: *N Engl J Med* 1997; 337: 1859).
112 Campeau L, Hunninghake DB, Knatterud GL et al. Aggressive cholesterol lowering delays saphenous vein graft atherosclerosis in women, the elderly, and patients with associated risk factors. NHLBI post coronary artery bypass graft clinical trial. Post CABG Trial Investigators. *Circulation* 1999; 99: 3241–3247.
113 Channon KM, Fulton GJ, Davies MG et al. Modulation of tissue factor protein expression in experimental venous bypass grafts. *Arterioscler Thromb Vasc Biol* 1997; 17: 1313–1319.
114 Chen S, Wilson J, Muller D. Adenovirus-mediated gene transfer of soluble vascular cell adhesion molecule to porcine interposition vein grafts. *Circulation* 1994; 89: 1922–1928.
115 Channon KM, Fulton GJ, Gray JL et al. Efficient adenoviral gene transfer to early venous bypass grafts: comparison with native vessels. *Cardiovasc Res* 1997; 35: 505–513.
116 Rollins F, Nong Z, Pisvin S, Collen D. Adeno-associated virus-mediated gene transfer into rat carotid arteries. *Gene Therapy* 1997; 4: 757–761.
117 Arnold TE, Gnatenko D, Bahou WF. In vivo gene transfer into rat arterial walls with novel adeno-associated virus vectors. *J Vasc Surg* 1997; 25: 347–355.
118 Xiao X, Li J, Samulski RJ. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. *J Virol* 1996; 70: 8098–8108.
119 Fisher KJ, Jooss K, Alston J et al. Recombinant adeno-associated virus for muscle directed gene therapy. *Nat Med* 1997; 3: 306–312.
120 Davies MG, Huynh TT, Fulton GJ et al. G protein signalling and vein graft intimal hyperplasia: reduction of intimal hyperplasia in vein grafts by a G-betagamma inhibitor suggests a major role of G protein signalling in lesion development. *Arterioscler Thromb Vasc Biol* 1998; 18: 1275–1280.
121 Schwartz LB, Moawad J, Svensson EC et al. Adenoviral-mediated gene transfer of a constitutively active form of the retinoblastoma gene product attenuates neointimal thickening in experimental vein grafts. *J Vasc Surg* 1999; 29: 874–881.

Cambridge Books Online © Cambridge University Press, 2009

Pharmacological modulation of the inflammatory actions of platelets

Carla Cicala and Giuseppe Cirino

Department of Experimental Pharmacology, University of Naples 'Federico II', Naples, Italy

Cross-talk between inflammation and coagulation pathways

Blood clotting is a host defence mechanism that, together with inflammatory and repair processes, provides to preserve the integrity of the vascular system. There is much evidence for a cross-talk between inflammation and coagulation coming from both experimental and clinical evidence. In this respect, there is now a tendency to consider an inflammatory event as a prethrombotic status and to look for therapeutic agents that modulate both processes. The endothelium is the first site where the coagulation and inflammatory cascades interact. Under physiological conditions, vascular endothelium prevents coagulation and thrombosis by releasing inhibitors of platelet activation and potent vasodilators, such as prostacyclin (PGI_2) and nitric oxide, counteracting the effect of procoagulant factors. Endothelial cells also synthesize a number of negatively charged molecules, such as glycoproteins, glycosaminoglycans, chondroitin sulfate and heparan sulfate, which contribute to a non-thrombogenic surface. On the endothelial surface, thrombomodulin (TM), a high affinity receptor for thrombin is also expressed. The complex thrombin–TM activates protein C (PC), a vitamin K dependent glycoprotein that circulates in plasma as zymogen. Once PC is activated, it forms a complex with a vitamin K-dependent cofactor, Protein S (PS) on endothelium. Activated Protein C (APC) plays a major role in regulating the coagulation and fibrinolysis by inactivating factor Va and VIIIa and by inactivating tissue-plasminogen activator inhibitor (t-PAI). Thus, APC inhibits coagulation and stimulates the fibrinolytic pathway[1–4].

An endothelial protein C receptor (EPCR) has been identified and it seems to participate in the regulation of APC by binding to an exosite of APC and blocking the ability of APC to inhibit the factor Va[5,6]. In the plasma, APC is slowly inhibited by complex formation with PC inhibitor or alpha 1 antitrypsin; on the other hand, thrombin by activating the protein C pathway, acts as a feedback inhibitor of its own production[7]. It is clear that the presence of TM on endothelial surface and the integrity of the PC pathway are critical for the correct balance between coagulation and fibrinolysis. However, when the endothelium is damaged, this balance is lost, precipitating a procoagulant state[2,7,8].

In the complex events involved in inflammation, early produced cytokines strongly impair the balance between pro- and anti-coagulant factors. The major cytokines responsible for this impairment are TNFα and IL-1β which, by eliciting tissue factor (TF) production on endothelium and monocytes, provide an intravascular source of TF, causing activation of the extrinsic pathway of coagulation. At the same time, activation of Hageman factor by kallikrein leads to activation of the intrinsic coagulation pathway. Intrinsic and extrinsic pathways merge at a common point, e.g. the activation of factor X, and finally lead to fibrin formation. Fibrin clots, formed following activation of the coagulation system, has a temporary function; once damage has been repaired and blood loss arrested, the fibrin clot is removed and blood flow restored by activation of the fibrinolytic system. This latter phenomenon, known as fibrinolysis, also involves the release of substances from endothelial cells. In this case, the endothelium releases tissue plasminogen activator (t-PA), whose function is to convert plasminogen to plasmin; t-PA activity is modulated by tissue plasminogen activator inhibitor (t-PAI), also released by endothelial cells. The balance between t-PA and t-PAI is critical for the modulation of the fibrinolytic pathway[1,9,10].

All the effects so far discussed are up-regulated by cytokines; however, cytokines are also responsible for the

down-regulation of TM and PC expression. Once TM is down-regulated, thrombin does not bind to it and the PC pathway is not activated thereby the balance is shifted towards a procoagulant state which may lead to critical thrombosis[7].

Although it is not easy to dissect these two pathways that are so intimately related, the role of the coagulation system in several inflammatory disorders has been evaluated. There is much evidence that naturally occurring anticoagulants, such as APC, antithrombin III (AT III), TM and inhibitors of coagulation factors can improve symptoms of local and systemic inflammation. In particular, experimental sepsis has been considered as a paradigm to study the interplay between these two systems. PC activation plays a critical role in the host response to bacterial endotoxins and several studies have reported on the beneficial effect of low doses of APC on thrombosis or on disseminated intravascular coagulation (DIC) during endotoxemia. On the other hand, inhibition of PC by specific antibodies, before challenging animals with *E.coli* lipopolysaccharide, causes a more severe DIC[11,12]. Experimental evidence in animals has been confirmed by clinical studies demonstrating that APC supplementation has a beneficial role in human sepsis[4]. Furthermore, there are also data reporting a beneficial effect of ATIII supplementation in sepsis, based upon the observation that reduced ATIII levels in DIC are associated with increased mortality[13].

Another interaction between the inflammation and coagulation pathways is represented by proteases involved in coagulation, such as factor Xa and thrombin, that mediate several biological effects through activation of specific receptors, termed protease activated receptors (PARs). In particular, thrombin has been shown to play a role in several inflammatory events, to stimulate cell proliferation and cytokine production. Similarly, factor Xa has been shown to have a proinflammatory effect through activation of its receptor EPR-1 (effector cell protease receptor-1)[14,15].

In the light of this finding, which assigns a role to proteases beyond their 'procoagulant' effects, it is easy to understand why these molecules could represent a target for new anti-inflammatory drugs.

Platelets in inflammation

Early evidence for the involvement of platelets in inflammation was already given in 1961 by Jancsò, who showed by microscopy that inflammation induced in rat paw by local injection of thrombin was accompanied by clumping of platelets together and to damaged endothelium, polymerization of fibrinogen into fibrin strands and localization of microthrombi in capillaries and lymphatics[16]. In 1968, Wiseman and colleagues demonstrated that carrageenin-induced edema in the rat paw was characterized by fibrin deposition, inhibited by anticoagulant agents and increased by systemic administration of thrombin[17]. It is known that fibrin activates platelets through binding with specific receptors on the platelet surface. Since fibrin formation is strictly linked to platelet activation, inflammatory reactions that involve fibrin polymerization are also characterized by clumping of platelets in damaged organs.

Normally, platelets circulate freely in the blood, by mechanisms involving non-adhesive properties of endothelial cells[18,19]. However, when endothelium is damaged, such as during inflammation, the exposure of collagen fibres of the subendothelium, together with the loss of antihemostatic properties of endothelium at the site of injury, promotes platelet adhesion on to endothelium with subsequent activation. Platelet adhesion to endothelium is mediated by adhesion molecules expressed on both endothelial cells and platelets following stimulation by cytokines. P-selectin receptors expressed on the surface of both activated platelets and endothelial cells, as well as the presence of a receptor protein for P-selectin on monocytes and neutrophils promotes interactions among these cells. The protein P-selectin is normally present on the external membrane of platelet α-granules and of Weibel–Palade bodies of endothelial cells, and translocates to the cell membrane soon after cell activation by thrombin or other platelet agonists[20,21]. Hence, P-selectin plays a pivotal role in regulating interactions among platelets, endothelial cells, monocytes and neutrophils. Indeed, appearance of P-selectin on platelets and endothelial cells precedes other adhesion molecule expression, and it is feasible that interactions among platelets, endothelial cells and neutrophils contributes to platelet translocation at the site of injury and to the release of proinflammatory mediators in the early phase of inflammation. Moreover, P-selectin can induce the expression of tissue factor on monocytes participating, in this way, to amplify activation of the coagulation cascade[22]. In this context, P-selectin plays an important role in the orchestration of the inflammatory response[23].

Platelets have been shown to contain a number of cytokines that contribute to modifying endothelial function and participating in the spreading of an inflammatory lesion. Stimulated platelets express IL-1β on their surface, providing an early source of this cytokine at the site of injured endothelium. Through IL-1β, activated platelets induce the expression of intercellular adhesion molecule 1 (ICAM-1), IL-6, IL-8 and granulocyte monocyte-colony

Cambridge Books Online © Cambridge University Press, 2009

stimulating factor (GM-CSF) in endothelial cells. These cytokines all contribute to leukocyte attraction and adhesion to endothelium[24,25]. While IL-1 remains associated to platelet membrane, other cytokines are stored in α-granules and released upon platelet activation. Transforming growth factor beta (TGF-β) is an α-granule product that promotes chemotaxis, inhibits endothelial cell proliferation, enhances synthesis of PAI-1 and increases the production of endothelin–1[26–28]. There is evidence that injection of TGFβ subcutaneously to experimental animals leads to granuloma formation suggesting a role for TGFβ in wound healing and tissue proliferation[29]. Alpha granules also contain PDGF that is the major mitogen associated with platelets and it plays a role in pathogenesis of atherosclerosis, by stimulating proliferation of arterial smooth muscle cells[30,31]. Moreover, PDGF plays an important role in the generation of granulation tissue during wound repairing, in inflammatory reactions and proliferative responses[32,33]. Another cytokine contained in α-granules is the platelet factor 4 (PF4). PF-4 can neutralize the anticoagulant activity of heparin or compete for the binding to AT III[34,35] and stimulates histamine release from basophils[36]. Platelet α-granules are also a source of the secreted form of phospholipase A_2 (PLA_2; 14 kDa), an enzyme that has been found in several inflammatory exudates[37] and shown to be proinflammatory[38] or to exacerbate an ongoing inflammation[39,40].

As well as being factors secreted by α-granules, platelets also release the source of other substances, mostly amines released following degranulation of dense granules. In this respect, platelets, as inflammatory cells, have been compared to mast cells. Like mast cells, upon activation they degranulate and release a wide array of inflammatory mediators that can cause flogosis when injected locally in experimental animals[41,42].

Platelets also synthesize lipid mediators *ex novo*, such as eicosanoids and platelet activating factor (PAF). Among eicosanoids, thromboxane A_2 (TXA_2) is the major product of the metabolism of arachidonic acid in platelets, and is synthesized following platelet activation and causes platelet aggregation and degranulation. At the site of injury, TXA_2 formed by activated platelets contributes to stabilizing platelet aggregates. TXA_2 has been also shown to cause smooth muscle contraction and thus it provides local vasoconstriction[43].

PAF is a lipid mediator produced by platelets and a wide array of inflammatory cells, and it is one of the most potent inflammatory mediators of lipid origin. Injection of PAF into experimental animals causes a variety of responses, some of them involve platelet activation, while others are independent of platelets. PAF has been shown to be a potent bronchoconstrictor agent; given intravenously to experimental animals, PAF-induced bronchoconstriction is dependent upon platelet activation, indeed in animals depleted of platelets, or in animal species whose platelets are unresponsive to PAF, this reaction is prevented. However, PAF also causes airway hyper-responsiveness when given by aerosol[44,45] and it increases vascular permeability in the acute inflammatory reaction[46,47], in sepsis[48] and in gastrointestinal inflammation[49,50]. PAF is also an eosinophil chemoattractant[42].

Cooperation between platelets and polymorphonuclear leukocytes for production of proinflammatory mediators[51–53] and between platelets and monocytes for protection against parasites[54,55] has also been shown. However, platelets contribute to inflammation not only via the release of a wide range of mediators, but also via the ability to kill mycobacteria and bacterial endotoxins, providing protection against invading organisms[56,57].

Direct evidence for the involvement of platelets in inflammation comes from studies performed using labelled platelets that have shown platelet accumulation in the inflamed area after local administration of an irritant[58]. An increased platelet response to aggregating agent has also been shown for platelets obtained from rats injected in the paw with carrageenin[59] or from humans affected by asthma[60], psoriasis[61] or intestinal tubercolosis[62].

There is much evidence for an involvement of platelets in sepsis. In particular, a decrease in platelet count has been shown several hours after endotoxin administration, and thrombocytopenia has been shown to be correlated to clumping of platelets in liver and spleen[63,64]; platelet responsiveness following endotoxin administration to rats is reduced[65]. Anticoagulant agents have been shown to ameliorate shock symptoms as well as to prevent thrombocytopenia, outlining the importance of platelets in the organ damage associated with bacterial endotoxemia[11,66].

Furthermore, platelet depletion has been shown to protect animals from both allergic and non-allergic inflammation[41]. Moreover, it has been demonstrated that paw edema development in mice is inhibited after splenectomy[67].

Platelets also represent a surface for clotting factor activation and they are required for optimal expression of clotting activity. Factor V released from α-granules binds to platelet surface and, together with anionic phospholipids exposed, functions as a binding site for factor Xa. An analogous system involves the binding of factor IX and factor VIII that in presence of membrane phospholipids converts factor X to Xa on platelet surface. Thus, platelets are involved in inflammation either as a source of inflammatory mediators and as a surface for coagulation. Moreover, by interacting with other cells platelets contribute to amplifying proinflammatory signals[2,8].

Cambridge Books Online © Cambridge University Press, 2009

In the light of this knowledge, the mechanism by which several agents are beneficial in inflammation has been revised and it has been now recognized that inhibition of platelet activation or the neutralization of platelet derived mediators might be useful in preventing tissue damage during an inflammatory state.

Platelet thrombin receptor(s)

Thrombin signalling to cells is mediated through a family of G-protein coupled receptors, designated as PARs (*protease activated receptors*). The first identified was the thrombin receptor PAR-1 that presents, together with other PARs, a peculiar mechanism of autoactivation. Following proteolytic cleavage by thrombin, between a residue of Arg-41 and Ser-42, a new N-terminus peptide is unmasked which acts as a tethered ligand. Synthetic peptides containing a minimum of five residues (SFLLR) of the tethered ligand can activate the receptor, even in the absence of proteolysis. The mechanism of PAR-1 activation is irreversible and the tethered ligand generated cannot diffuse away from the receptor. Up to now, four PARs have been identified: PAR-1, PAR-3 and PAR-4 can be activated by thrombin, while PAR-2 is activated by trypsin, tryptase and factor Xa[68].

Thrombin receptors on platelets and the requirement of receptor activation to trigger platelet responses vary among species. Evidence that PAR-1 blocking antibodies inhibited human platelet activation induced by low, but not by high, thrombin concentrations, and that PAR-1 deficient mice responded like wild type mice to thrombin, has led to the discovery of PAR-3 and PAR-4[69,70]. Human platelets have both PAR-1 and PAR-4[71]; in contrast, mouse platelets express PAR-3 and PAR-4, but not PAR-1[72–74]. In human PAR-1 and in mouse PAR-3 are high affinity thrombin receptors, while in both species PAR-4 is a low affinity thrombin receptor. Recent evidence suggests that either in humans or in mice activation of both receptors is necessary to trigger a full platelet response[71]. PAR-1 activation causes an early, transient response, while PAR-4 activation is responsible for a late, prolonged signal[75]. The existence of two different thrombin receptors on human platelets might explain why synthetic peptides activating PAR-1 or PAR-4 are not able to trigger the same platelet response caused by thrombin. Evidence that peptides activating PAR-1 inhibit thrombin-mediated rat platelet aggregation suggests the presence of a modified PAR-1 in rats rather than a total absence of this type of receptor[76].

Platelet activation by thrombin has been extensively studied and it includes aggregation, degranulation and stimulation of platelet procoagulant activity. Besides thrombin, other platelet agonists, such as collagen and calcium ionophore, A23187, stimulate platelet procoagulant activity, measured as an increase of platelet phosphatidylserine and posphatidylethanolamine exposure[77].

On human platelets, PAR-1 and PAR-4 contribute differently to mediate thrombin effects; PAR-1 activation by thrombin is mainly responsible for platelet activation, calcium flux and clotting system activation, while PAR-4 activation leads to a less potent platelet response[78]. In this respect, since activation of the coagulation cascade is an important event in the development of inflammation, it is feasible that PAR-1 more than PAR-4 plays a major role in inflammation. This hypothesis is consistent with experimental evidence demonstrating a role for PAR-1 in the inflammatory response triggered by thrombin[15].

The knowledge on the proinflammatory effect of thrombin has been greatly improved only following identification and cloning of the thrombin receptor and its activation by small synthetic peptides mimicking the sequence of the tethered ligand. In 1996, it was possible to demonstrate that thrombin acts as a mediator of inflammation, through activation of PAR-1[79]. Further studies have shown the involvement of PAR-1 even in other proinflammatory responses to thrombin. Indeed, PAR-1 mediates thrombin-induced bronchoconstriction in guinea-pig but not in rats, whose platelets lack PAR-1[80].

In 1990 Ferrer-Lopez et al. demonstrated that cathepsin G released by activated neutrophils stimulates human platelets and reduces platelet response to thrombin[81]. The mechanism at the basis of the cathepsin G effect seems to be due to its ability to cleave PAR-1, creating a ‘disarmed’ receptor unable to be activated by thrombin[82]. Recently, it has been shown that PAR-4 is involved in the cathepsin G effect; PAR-4 activated by cathepsin G released by neutrophils, mediates human platelet secretion and aggregation[83]. This finding suggests a role for PAR-4 in inflammation, by mediating neutrophil–platelet interaction at the site of injury.

An interaction between PAR-1 and aprotinin has been recently demonstrated[84]. It has been shown that aprotinin inhibits thrombin-induced platelet activation by preventing PAR-1 cleavage by thrombin. There is much literature on the protective role of aprotinin in diseases such as sepsis. Thus, in the light of these new findings, it is feasible that the mechanism involving PAR-1 inhibition is at the basis of the aprotinin protective effect.

Cambridge Books Online © Cambridge University Press, 2009

Drugs affecting inflammatory functions of platelets

Evidence that platelets are involved in inflammation has led to platelets being considered as targets for anti-inflammatory agents.

Antiserototinergic and antihistaminergic drugs

Biogenic amines, histamine and serotonin, participate in the vascular events characteristic of the early phase of an immune or non-immune inflammatory reaction, both in humans and in experimental animals[85–87].

Although mast cells have been implicated as the main source, there is much evidence that also platelets are source of biogenic amines at the site of injury. Platelet accumulation at the site of injury has been demonstrated either in carrageenin-induced edema, or in zymosan-induced pleurisy. Platelet derived serotonin has been implicated not only in acute but also in chronic inflammation, both in humans and in experimental animals[85,86], as well as in pain associated with inflammation[88,89].

Among different species, release of histamine by platelets has been demonstrated in pig, rabbit and humans[90,91]. Platelet histamine content has been shown to be increased in humans affected by peripheral vascular disorders[92].

Antiserotinergic and antihistaminergic drugs are effective in the early phase of inflammation either in carrageenin-induced rat paw edema or in zymosan-induced rat pleurisy, at a time when indomethacin is not effective[87,93] and when platelet accumulation occurs at sites of inflammation. Thus, it is feasible that in the early stage of an inflammatory event, platelets are the main source of histamine and serotonin.

There are several lines of evidence that platelet functions are altered in asthmatics and in patients suffering from atopic diseases. Platelet activation occurs during asthma and platelet hyper-responsiveness is a common feature of asthmatic subjects[60,94]. Experimental evidence has shown that disodium cromoglycate is able to inhibit PAF-induced airway hyper-reactivity in asthmatic subjects and also to inhibit human platelet aggregation induced by ADP and collagen[95]. This effect has been suggested to be involved in its beneficial role in the treatment of asthma. Further studies have shown that ketotifen, whose mechanism is similar to that of sodium cromoglycate, inhibits both rabbit and human platelet activation[96]. Nedocromil sodium reduces platelet hyper-reactivity from ASA-induced asthmatic subjects[94].

Hirudin and Hirulog

The prototype of a direct thrombin inhibitor is hirudin, first isolated from saliva of the leech *Hirudo medicinalis* [98]. Hirudin inhibits thrombin by binding to both anionic and catalytic sites, that recognise fibrinogen and the platelet surface. Several hirudin-like peptides have been synthesized, among these hirulog has been extensively described as a thrombin inhibitor[99,100]. Hirudin and hirulog have been shown to be beneficial in several inflammatory disorders[101] and to inhibit inflammatory effects of thrombin on endothelial cells[102]. More recently, it has been shown that PEG-hirudin inhibits collagen-induced arthritis in mouse, demonstrating a role for thrombin also in an inflammatory reaction with an immunologic basis.[103]

In rat experimental endotoxin shock, hirulog inhibits thrombocytopenia induced by bacterial endotoxin, suggesting that inhibition of platelet activation might partially contribute to its beneficial effect.[66]

Heparin

Heparin and other anticoagulants were firstly described to be beneficial as anti-inflammatory agents in 1968 by Wiseman and Chang[17], who demonstrated that several anticoagulant agents can inhibit carrageenin induced rat paw edema, while thrombin had a potentiating effect. Subsequently, anticoagulant agents have been extensively used as anti-inflammatory agents, even though the mechanism at the basis of this effect is unclear. There is fairly recent evidence for inhibition of thrombin platelet stimulation by high and low molecular weight heparins and this mechanism might be involved in the anti-inflammatory effect exerted by these molecules[104].

NSAIDs

The most used anti-inflammatory drugs all over the world are non-steroid anti-inflammatory drugs (NSAIDs). Among them, aspirin is widely used for its analgesic, anti-inflammatory and antithrombotic properties. Compared to other drugs, aspirin has the ability to irreversibly inhibit platelet COX-1 through the acetylation of the enzyme.

Non-selective COX-1 and COX-2 inhibitors are the most widely used anti-inflammatory drugs. Although the research in the field has developed COX-2 selective inhibitors that have been shown to be devoid of gastrotoxicity, there is evidence that in some models of inflammation, COX-1 inhibitors appear to be more effective than selective COX-2 inhibitors. A study performed by testing the anti-inflammatory effect of different COX-1 and COX-2

Cambridge Books Online © Cambridge University Press, 2009

inhibitors on rat experimental pleurisy showed that the most effective in reducing cell influx and exudation were aspirin and piroxicam, but not the more COX-2 selective inhibitors, NS398 and nimesulide[105]. Since platelets possess only COX-1, it is likely that platelet COX-1 inhibition is essential for some of the anti-inflammatory effects displayed by NSAIDs.

Concluding remarks

Inflammation is undoubtedly linked to coagulation and recent work suggests that drugs targeting the coagulation pathway may provide novel therapeutic approaches for inflammatory conditions.

References

1 Preissner KT. Physiological role of vessel wall related antithrombotic mechanisms: contribution of endogenous and exogenous heparin-like components to the anticoagulant potential of the endothelium. *Haemostasis* 1990; 20: 30–49.
2 Bloom AL. Physiology of blood coagulation 1990; 20: 14–29.
3 Esmon CT. Cell mediated events that control blood coagulation and vascular injury. *Ann Rev Cell Biol* 1993; 9: 1–26.
4 Esmon C. The protein C pathway. *Crit Care Med* 2000; 28 (Suppl.9): S44–S48.
5 Fukudome K, Esmon CT. Molecular cloning and expression of murine and bovine endothelial cell protein C/activated protein C receptor (EPCR). *J Biol Chem* 1995; 270: 5571–5577.
6 Rock G, Wells P. New concepts in coagulation. *Crit Rev Clin Ab Sci* 1997; 34: 475–501.
7 Esmon CT, Taylor FB Jr, Snow R. Inflammation and coagulation: linked processes potentially regulated through a common pathway mediated by protein C. *Thromb Haemost* 1991; 66: 160–165.
8 Cicala C, Cirino G. Linkage between inflammation and coagulation: an update on the molecular basis of the crosstalk. *Life Sci* 1998; 62: 1817–1824.
9 Lijnen HR. Pathophysiology of the plasminogen/plasmin system. *Int J Clin Lab Res* 1996; 26: 1–6.
10 Kaplan AP, Kusumama J, Shibayama Y, Reddigari S, Ghebrehiwet B, Silverberg M. The intrinsic coagulation/kinin-formin cascade. Assembly in plasma and cell surfaces in inflammation. *Adv Immunol* 1997; 66: 225–272.
11 Taylor FB Jr, Chang A, Esmon CT, D'Angelo A, Vigano-D'Angelo S, Blick KE. Protein C prevents coagulopathic and lethal effects of *Escherichia coli* infusion in the baboon. *J Clin Invest* 1987; 79: 918–925
12 Taylor FB, Stearns-Kurosawa DJ, Kurosawa S et al. The endothelial cell protein C receptor aids in host defense against *Escherichia coli* sepsis. *Blood* 2000; 95: 1680–1686.
13 Fourier F, Chopin C, Goudemand J et al. Septic shock, multiple organ failure, and disseminated intravascular coagulation: compared patterns of antithrombin III, protein C and protein S deficiencies. *Chest* 1992; 101: 816–823.
14 Cirino G, Cicala C, Bucci M et al. Factor Xa as an interface between coagulation and inflammation. *J Clin Invest* 1997; 99: 2446–2451.
15 Cirino G, Napoli C, Bucci M, Cicala C. Inflammation and coagulation network: are serine protease the knot? *Trends Pharmacol Sci* 2000; 21: 170–172.
16 Jancsò N. Inflammation and the inflammatory mechanisms. *J Pharm Pharmacol* 1961; 13: 577–594.
17 Wiseman EH, Chang Y. The role of fibrin in the inflammatory response to carrageenin. *J Pharmacol Exp Ther* 1968; 159: 206–209.
18 Houston DS, Robinson P, Gerrard JM. Inhibition of intravascular platelet aggregation by endothelium-derived relaxing factor: reversal by red blood cells. *Blood* 1990; 76: 953–958.
19 Moncada S, Higgs A. The L-arginine–nitric oxide pathway. *N Engl J Med* 1993; 329: 2002–2012.
20 McEver RP. GMP-140: a receptor for neutrophils and monocytes on activated platelets and endothelium. *J Cell Biochem* 1991; 45: 156–161.
21 McEver RP. Role of selectins in leukocyte adhesion to platelets and endothelium. *Ann NY Acad Sci* 1994; 714: 185–189.
22 Celi A, Pellegrini G, Lorenzet R et al. P-selectin induces the expression of tissue factor on monocytes. *Proc Natl Acad Sci USA* 1994; 91: 8767–8771.
23 Lorant DE, Topham MK, Whatley RE et al. Inflammatory roles of P-selectin. *J Clin Invest* 1993; 92: 559–570.
24 Hawrylowicz CM, Howells GL, Feldmann M. Platelet-derived interleukin 1 induces human endothelial adhesion molecules expression and cytokine production. *J Exp Med* 1991; 174: 785–790.
25 Hawrylowicz CM. Viewpoint: a potential role for platelet derived cytokines in the inflammatory response. *Platelets* 1993; 4: 1–10.
26 Magyar-Lehman S, Bohlen P. Purification of platelet-derived endothelial growth inhibitor and its characterization as tranforming growth factor-β type 1. *Experientia* 1992; 48: 374–379.
27 Slivka SR, Loskutoff DJ. Platelets stimulate endothelial cells to synthesize type 1 plasminogen activator inhibitor. Evaluation of the role of transforming growth factor β. *Blood* 1991; 77: 1013–1019.
28 Murata S, Matsumura Y, Takada K, Asai Y, Takaoka M, Morimoto S. Role of transforming growth factor beta-1 on platelet-induced enhancement of endothelin-1 production in cultured vascular endothelial cells. *J Pharmacol Exp Ther* 1995; 274: 1524–1530.
29 Mustoe TA, Pierce GF, Thomason A, Gramates P, Sporn MB, Deuel TF. Accelerated healing of incisional wounds in rats induced by transforming growth factor β. *Science* 1987; 237: 1333–1336.
30 Friedman RJ, Stemerman MB, Wenz B. The effect of thrombo-

Cambridge Books Online © Cambridge University Press, 2009

cytopenia on experimental atherosclerotic lesion formation in rabbits: smooth muscle cell proliferation and reendothelialization. *J Clin Invest* 1977;60: 1191–1201.

31 Ross R. Platelet-derived growth factor. *Lancet* 1989; 1(8648); 1179–1182.

32 Deuel TF, Senior RM, Huang JS, Griffin GL. Chemotaxis of monocytes and neutrophils to platelet-derived growth factor. *J Clin Invest* 1982; 69: 1046–1049.

33 Deuel TF, Kawahara RS, Mustoe TA, Pierce GF. Growth factors and wound healing: platelet-derived growth factor as a model cytokine. *Annu Rev Med* 1991; 42: 567–584.

34 Busch C, Dawes J, Pepper DS, Wateson A. Binding of platelet factor 4 to cultured human umbilical vein endothelial cells. *Thromb Res* 1980; 19: 129–137.

35 Marcum JA, McKenney JB, Rosenberg RD. Acceleration of thrombin-antithrombin complex formation in rat hindquarters via heparin-like molecules bound to the endothelium. *J Clin Invest* 1984; 74: 341–350.

36 Brindley LL, Sweet JM, Goetzl EJ. Stimulation of histamine release from human basophils by human platelet factor 4. *J Clin Invest* 1983; 72: 1218–1223.

37 Murakami M, Kudo I, Inoue K. In vivo release and clearance of rat platelet phospholipase A_2. *Biochim Biophys Acta* 1989; 1005: 270–276.

38 Cirino G, Peers SH,Wallace JL, Flower RJ. A study on phospholipase A_2 induced oedema in rat paw. *Eur J Pharmacol* 1989; 166: 505–510.

39 Cirino G, Cicala C, Sorrentino L. Human recombinant platelet phospholipase A_2 exacerbates poly-L-arginine induced rat paw edema. *Inflammation* 1994; 18: 59–66.

40 Murakami M, Kudo I, Nakamura H, Yokoyama Y, Mori H, Inoue K. Exacerbation of rat adjuvant arthritis by intradermal injection of purified mammalian 14-kDa group II phospholipase A_2. *FEBS Lett* 1990; 268: 113–116.

41 Page CP. Platelets as inflammatory cells. *Immunopharmacology* 1989; 17: 51–59.

42 Mannaioni PF, Di Bello MG, Masini E. Platelets and inflammation: role of platelet-derived growth factor, adhesion molecules and histamine. *Inflamm Res* 1997; 46: 4–18.

43 Packam MA. Role of platelets in thrombosis and hemostasis. *Can J Physiol Pharmacol* 1994; 72: 278–284.

44 Vargaftig BB, Lefort J, Chignard M, Benveniste J. Platelet activating factor induces a platelet-dependent bronchoconstriction unrelated to the formation of prostaglandin derivatives. *Eur J Pharmacol* 1980; 65: 185–192.

45 Coyle AJ, Spina D, Page CP. PAF-induced bronchial hyperresponsiveness in the rabbit: contribution of platelets and airway smooth muscle. *Br J Pharmacol* 1990; 101: 31–38.

46 Silva PM, Cordeiro RS, Martins MA, Henriques MG, Vargaftig BB. Platelet involvement in rat paw edema induced by 2-methoxy-PAF. *Inflammation* 1986; 10: 393–401.

47 Hayashi M, Kimura J, Yamaki K et al. Detection of platelet activating factor in exudates of rats with phorbol myristate acetate-induced pleurisy. *Thromb Res* 1987; 48: 299–310.

48 Graham RM, Strahan ME, Norman KW, Watkins DN, Sturm MJ, Taylor RR. Platelet and plasma platelet-activating factor in sepsis and myocardial infarction. *J Lipid Mediat Cell Signal* 1994; 9: 167–182.

49 Rosam AC, Wallace JL, Whittle BJR. Potent ulcerogenic actions of platelet activating factor on the stomach. *Nature* 1986; 319: 54–56.

50 Sobhani I, Hochlaf S, Denizot Y et al. Raised concentration of platelet activating factor in colonic mucosa of Crohn's disease patients. *Gut* 1992; 33: 1220–1225.

51 Marcus AJ, Safier LB, Ullman HL et al. 12S,20-dihydroxyicosatetraenoic acid: a new icosanoid synthesized by neutrophils from 12S-hydroxyicosatetraenoic acid produced by thrombin- or collagen-stimulated platelets. *Proc Natl Acad Sci USA* 1984; 81: 903–907.

52 Edenius C, Haeggstrom J, Lindgren JA. Transcellular conversion of endogenous arachidonic acid to lipoxins in mixed human platelet-granulocyte suspension. *Biochem Biophy Res Commun* 1988; 157: 801–807.

53 Palmentier R, Borgeat P. Thrombin activated platelets promote leukotriene B4 synthesis in polymorphonuclear leukocytes stimulated by physiological agonists. *Br J Pharmacol* 1991; 103: 1909–1916.

54 Joseph M, Auriault C, Capron A, Vorng H, Vieus P. A new function for platelets: IgE-dependent killing of Schistosomes. *Nature* 1983; 302: 810–812.

55 Pancré V, Monte D, Delanoye A, Capron A, Auriault C. Interleukin-6 is the main mediator of the interaction between monocytes and platelets in the killing of *Schistosoma mansoni*. *Eur Cytokines Netw* 1990; 1: 15–19.

56 Des Prez RM, Horowitz HI, Hook EW. Effects of bacterial endotoxin on rabbit platelets. I. Platelet aggregation and release of platelet factors in vitro. *J Exp Med* 1961; 114: 857–874.

57 Semeraro N, Lattanzio A. Interaction of platelets with bacterial endotoxins. *Agents Actions* 1983; 13: 461–469.

58 Vincent JE, Bonta IL, Zijlstra FJ. Accumulation of blood platelets in carrageenin rat paw oedema. Possible role in the inflammatory process. *Agents Actions* 1978; 3: 291–295.

59 Srivastava R, Srimal RC. Amplification of platelet response during acute inflammation in rats. *Biochem Pharmacol* 1990; 40: 357–363.

60 Gresele P, Dottorini M, Selli ML et al. Altered platelet function associated with the bronchial hyperresponsiveness accompanying nocturnal asthma. *J Allergy Clin Immunol* 1993; 91: 894–902.

61 Hayashi S, Shimizu I, Miyauchi H, Watanabe S. Increased platelet aggregation in psoriasis. *Acta Derm Venereol* 1985; 65: 258–262.

62 Sarode R, Bhasin D, Marwaha N et al. Hyperaggregation of platelets in intestinal tubercolosis. Role of platelets in chronic inflammation. *Am J Hematol* 1995; 48: 52–54.

63 Endo Y, Nakamura M. The effect of lipopolysaccharide, interleukin-1 and tumor necrosis factor on the hepatic accumulation of 5-hydroxytryptamine and platelets in the mouse. *Br J Pharmacol* 1992; 105: 613–619.

Cambridge Books Online © Cambridge University Press, 2009

64 Itoh H, Cicala C, Douglas GJ, Page CP. Platelet accumulation induced by bacterial endotoxin in rats. *Thromb Res* 1996; 83: 405–419.
65 Cicala C, Santacroce C, Itoh H, Douglas GJ, Page CP. A study on rat platelet responsiveness following endotoxin intravenous administration in rats. *Life Sci* 1997; 60: PL31–38.
66 Cicala C, Bucci M, Maraganore JM, Cirino G. Hirulog effect in rat endotoxin shock. *Life Sci* 1995; 57: PL 307–313.
67 Franco CD, Spillert CR, Donohoe MJ, Lazaro EJ. Effect of splenectomy on acute inflammation. *Am Surg* 1986; 52: 237–239.
68 Dery O, Corvera CU, Steinhoff M, Bunnett NW. Proteinase-activated receptors: novel mechanisms of signalling by serine proteases. *Am J Physiol* 1998; 274: C1429–C1452.
69 Hung DT, Vu THK, Wheaton VI, Ishii K, Coughlin SR. Cloned platelet thrombin receptor is necessary for thrombin-induced platelet activation. *J Clin Invest* 1992; 89: 1350–1353.
70 Brass LF, Vassallo RR, Belmonte E, Ahuja M, Cichowski K, Hoxie JA. Structure and function of the human platelet thrombin receptor. *J Biol Chem* 1992; 267: 13795–13798.
71 Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J Clin Invest* 1999; 103: 879–887.
72 Ishihara H, Connolly AJ, Zeng D et al. Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature* 1997; 278: 502–506.
73 Xu WF, Andersen H, Whitmore TE et al. Cloning and characterization of human protease-activated receptor 4. *Proc Natl Acad Sci USA* 1998; 95: 6642–6646.
74 Kahn ML, Zheng YW, Huang W et al. A dual thrombin receptor system for platelet activation. *Nature* 1998; 394: 690–694.
75 Covic L, Gresser AL, Kuliopulos A. Biphasic kinetics of activation and signalling for PAR 1 and PAR 4 thrombin receptors in platelets. *Biochemistry* 2000; 39: 5458–5467.
76 Ruef J, Kacharava A, Pohl J, Runge MS. Indications for the presence of an atypical protease-activated receptor on rat platelets. *Ann Hematol* 2000; 79: 604–611.
77 Coughlin SR. Thrombin signalling and protease-activated receptors. *Nature* 2000; 407: 258–264.
78 Andersen H, Greenberg DL, Fujikama K, Xu W, Chung DW, Davie EW. Protease- activated receptor 1 is the primary mediator of thrombin stimulated platelet procoagulant activity. *Proc Natl Acad Sci USA* 1999; 96: 11189–11193.
79 Cirino G, Cicala C, Bucci M, Sorrentino L, Maraganore JM, Stone SR. Thrombin function as an inflammatory mediator through activation of its receptor. *J Exp Med* 1996; 183: 821–827.
80 Cicala C, Bucci M, De Dominicis G, Harriot P, Sorrentino L, Cirino G. Bronchoconstrictor effect of thrombin and thrombin receptor activating peptide in guinea pigs in vivo. *Br J Pharmacol* 1999; 126: 478–484.
81 Ferrer-Lopez P, Renesto P, Schattner M, Bassot S, Laurent P, Chignard M. Activation of human platelets by C5a-stimulated neutrophils: a role for cathepsin G. *Am J Physiol* 1990; 258: C1100–C1107.
82 Kinlough-Rathbone RL, Perry DW, Rand ML, Packman MA. Effect of cathepsin G pretreatment of platelets on their subsequent responses to aggregating agents. *Thromb Res* 1999; 95: 315–323.
83 Sambrano GR, Huang W, Faruqi T, Mahrus S, Craik C, Coughlin SR. Cathepsin G activates protease-activated receptor-4 in human platelets. *J Biol Chem* 2000; 275: 6819–6823.
84 Poullis M, Manning R, Laffan M, Haskard DO, Taylor KM, Landis RC. The antithrombotic effect of aprotinin: actions mediated via the protease activated receptor-1. *J Thorac Cardiovasc Surg* 2000; 120: 370–378.
85 Yeatts RP, Turner R, Collins R, Kaufmann J, Mashburn H. Soluble and insoluble immune complex-platelet interactions in rheumatoid inflammation. *Ann Rheum Dis* 1978; 37: 421–427.
86 Little CH, Stewart AG, Fennessy MR. Platelet serotonin release in rheumatoid arthritis: a study in food-intolerant patients. *Lancet* 1983; 2(8345): 297–299.
87 Ameisen JC, Meade R, Askenase PW. A new interpretation of the involvement of serotonin in delayed-type hypersensitivity. Serotonin-2 receptor antagonists inhibit contact sensitivity by an effect on T cells. *J Immunol* 1989; 142: 3171–3179.
88 Hong Y, Abbott FV. Behavioural effect of intraplantar injection of inflammatory mediators in the rat. *Neuroscience* 1994; 63: 827–836.
89 Abbott FV, Hong Y, Blier P. Activation of 5HT2A receptors potentiates pain produced by inflammatory mediators. *Neuropharmacology* 1996; 35: 99–110.
90 Da Prada M, Pletscher A, Tranzer JP, Knuchel H. Subcellular localization of 5-hydroxytryptamine and histamine in blood platelets. *Nature* 1967; 216: 1315–1317.
91 Fukami MH, Holmsen H, Ugurbil K. Histamine uptake in pig platelets and isolated dense granules. *Biochem Pharmacol* 1984; 33: 3869–3874.
92 Gill DS, Barradas MA, Fonseca VA, Gracey L, Dandona P. Increased histamine content in leukocytes and platelets of patients with peripheral vascular disease. *Am J Pathol* 1988; 89: 622–626.
93 Imai Y, Hayashi M, Oh-ishi S. Involvement of platelet activating factor in zymosan induced rat pleurisy. *Lipids* 1991; 26: 1408–1411.
94 Johnson CE, Belfield PW, Davis S, Cooke NJ, Spencer A, Davies JA. Platelet activation during exercise induced asthma: effect of prophylaxis with cromoglycate and salbutamol. *Thorax* 1986; 41: 290–294.
95 Garcia Mesa M, Arruzazabala Valmana ML. Effect of ketotifen and disodium cromoglycate on human platelet aggregation. *Allergol Immunopathol* 1989; 17: 33–34.
96 Devillier P, Anoux B, Lalau Keraly C, Landes A, Marsac J, Benveniste J. Inhibition of human and rabbit platelet activation by ketotifen. *Fundam Clin Pharmacol* 1990; 4: 1–9.
97 Marquette CH, Joseph M, Tonnel AB et al. The abnormal in vitro response to aspirin of platelets from aspirin-sensitive asthmatics is inhibited after inhalation of nedocromil

Cambridge Books Online © Cambridge University Press, 2009

sodium but not of sodium cromoglycate. *Br J Clin Pharmacol* 1990; 29: 525–531.

98 Markwardt F. Past, present and future of hirudin. *Haemostasis* 1991; 21: 11–26.

99 Maraganore JM, Bourdon P, Jablonski J, Ramachandran KL, Fenton II JW. Design and characterization of hirulogs: a novel class of a bivalent peptide inhibitors of thrombin. *Biochemistry* 1990; 29: 7095–7101.

100 Chari S, Chauhan V. Hirudin-based synthetic peptides as inhibitors of thrombin. *Sem Thromb Hemost* 1994; 20: 315–318.

101 Cunningham MA, Rondeau E, Chen X, Coughlin SR, Holdsworth SR, Tipping PG. Protease activated receptor-1 mediated thrombin-dependent, cell mediated renal inflammation in crescentic glomerulonephritis. *J Exp Med* 2000; 191: 455–462.

102 Prescott SM, Seeger AR, Zimmerman GA, McIntyre TM, Maraganore JM. Hirudin-based peptides block the inflammatory effects of thrombin on endothelial cells. *J Biol Chem* 1990; 265: 9614–9616.

103 Marty I, Peclat V, Kirdaite G, Salvi R, So A, Busso N. Amelioration of collagen-induced arthritis by thrombin inhibition. *J Clin Invest* 2000; 107: 631–640.

104 De Candia E, De Cristofaro R, Landolfi R. Thrombin-induced platelet activation is inhibited by high- and low-molecular-weight heparin. *Circulation* 1999; 99: 3308–3314.

105 Gilroy DW, Tomlinson A, Willoughby DA. Differential effect of inhibitors of cyclooxygenase (cyclooxygenase 1 and cyclooxygenase 2) in acute inflammation. *Eur J Pharmacol* 1998; 355: 211–217.

Cambridge Books Online © Cambridge University Press, 2009

Cambridge Books Online © Cambridge University Press, 2009

Part V

Therapy

Cambridge Books Online © Cambridge University Press, 2009

Cambridge Books Online © Cambridge University Press, 2009

Design of trials to evaluate antiplatelet agents

Shamir R. Mehta[1,2], John W. Eikelboom[3], and Salim Yusuf[1,2]

[1]Population Health Institute, McMaster University, Hamilton, Canada
[2]Division of Cardiology, Hamilton Health Sciences Corporation, McMaster University, Hamilton, Canada
[1,3]Department of Haematology, Royal Perth Hospital, Perth, Australia

Introduction

Recognition of the central role of platelets in the pathophysiology of cardiovascular disease has led to intensified efforts to develop more effective and safer antiplatelet strategies. Randomized trials and meta-analyses of those trials are currently our most powerful tools for evaluating the effects of these newer antiplatelet strategies[1–3]. However, careful consideration of key issues in design, conduct and interpretation of randomized controlled trials is essential in order that moderate but clinically worthwhile treatment differences are reliably detected[4–6].

This chapter will describe the key underlying principles of randomized trials, with particular emphasis on clinical trials of antiplatelet therapy.

Fundamental principles of randomized controlled trials (Table 68.1)

The question

The criteria for a good clinical trial are straightforward. Ask an important question and answer it reliably. Even the most meticulously designed randomized trial will be of little use and, perhaps wasteful, if the study question is not of major clinical or biological relevance. A good study question is one which has not yet been answered, is based on a strong biologic rationale, and is relevant to individual patient care or public health.

Table 68.1. Key aspects of sound randomized control trial design

Design feature	Potential benefits
Proper randomization	Ensures that both known and unknown potential confounders are balanced between the treatment groups
Unbiased ascertainment of outcomes	
Blinding of patient	Avoids loss of the placebo effect which may otherwise inflate the observed treatment effect
Blinding of caregiver	Avoids differences between treatment groups in administration of effective cointerventions, compliance, or reporting of symptoms
Unbiased analyses of results	
Minimizing loss to follow-up	Avoids bias due to differential loss to follow-up among the treatment groups
Intention to treat analysis	Minimizes biases due to imbalance between patients who receive and those who do not receive study treatment/intervention
Avoidance of data-derived subgroups	Avoids focusing on underpowered analyses which may be potentially biased and misleading
Complete reporting of results according to prespecified plan	Avoids selective emphasis on particular subgroups and investigator driven biases based on knowledge of trial results

Minimizing bias: the principles of sound clinical trial design

If randomized trials are to provide unbiased and reliable estimates of treatment effect, they must avoid systematic biases and minimize random error. The potential for systematic bias can be minimized through (i) proper generation of the randomization sequence and concealment of treatment allocation; (ii) unbiased ascertainment of outcomes; (iii) unbiased analysis of results; and (iv) basing decisions on the totality of information (all related data from all relevant properly randomized trials).

Proper randomization

Randomization is the most important method of minimizing moderate biases in a clinical trial. The purpose of randomization is to allocate patients so as to balance treatment groups with respect to both known and unknown prognostic variables, which makes it more likely that the observed differences in outcome between the groups are due to the intervention or treatment. Secondly, randomization ensures the validity of statistical tests which enables one to ascribe, under the null hypothesis, a probability distribution for the differences in study outcomes, and therefore allows one to calculate the significance level (*P*-value) of the observed outcome of the trial in question. Thirdly, properly concealed randomization eliminates selection bias since this ensures that patients and clinicians remain unaware of treatment allocation prior to registration or randomization[7]. Many forms of randomization (such as randomizing patients according to day of the week) are inadequate because they allow both patients and clinicians to predict to which treatment the patient will be allocated. Inadequate concealment of treatment allocation can lead to systematic exaggeration of estimates of treatment effect[8]. Secure methods involving use of prepackaged blinded treatment or central telephone or computer-based randomization are now widely used in major cardiovascular trials.

Unbiased ascertainment of outcomes

Objective and unbiased ascertainment of study outcomes is critically important to the validity of a clinical trial. Ascertainment of outcomes may be biased if the patient or those responsible for treatment and evaluation know which treatment is being used. This problem may well be avoided if both patients and clinicians (or outcome evaluator) are blinded to the randomised treatment allocation, i.e. the trial is double-blind. Uncertainty remains regarding the importance of blinding with conflicting empirical evidence from recent studies addressing this issue[9,10]. However, most would agree that blinding is important, particularly when there are subjective outcomes in a trial (i.e. refractory angina, clinical DVT and recurrence of unstable angina) or when there is a potential for differential use of cointerventions that may be influenced by knowledge of the randomized treatment allocation or imbalances in other therapies that might affect outcome. By contrast, in clinical trials with objective and irreversible outcomes (i.e. mortality, myocardial infarction), blinding may be less important. For example, several major thrombolytic trials (including ISIS-3, GISSI-2 or GUSTO-1) were unblinded but used mortality as their primary outcome. However, in these trials, knowledge of the randomised treatment allocation may still have resulted in differences in the use of cointervention (e.g. use of percutaneous coronary intervention or coronary artery bypass graft surgery), which, in turn, could theoretically affect mortality. However, comparisons of ancillary treatments in open vs. blind trials of the same question have not shown systematic differences. Meanwhile, blinding may be of particular importance when outcome events are subjective in nature since knowledge of the randomized treatment allocation in this setting could influence both outcome ascertainment and use of cointerventions.

In some clinical trials, it may not be feasible to blind patients (for example, in trials of surgery vs. medicine). In this setting, blinded assessment of outcomes by a central committee of adjudicators may reduce the likelihood of bias.

Unbiased analysis of results

There are four principles that guide the approach to the analysis of clinical trial in order to minimize bias and increase validity. First, the number of patients lost to follow-up must be minimized (ideally, there should be no patients lost to follow-up); secondly, an intention-to-treat analysis should ideally be utilized; thirdly, an overemphasis on data-derived subgroup analyses should be avoided; and finally, complete reporting of the results of the trial according to a prespecified statistical analysis plan should be employed.

Patients who are lost to follow-up may differ from those who are not lost to follow-up in terms of their response to treatment and prognosis. If the proportion of patients lost to follow-up was randomly distributed among different treatment allocation groups, no bias would be expected. However, loss to follow-up is unlikely to be a random event. Therefore, complete follow-up (or near complete follow-up) is critical to ensure the validity of the results of a study. A similar rationale underlies the principle of analysis by intention-to-treat[6]. Patients who do not receive the allo-

Cambridge Books Online © Cambridge University Press, 2009

cated treatment (for whatever reason) may differ systematically from patients who receive the allocated treatment, and these selection processes may differ between treatment groups. Any potential biases resulting from this imbalance can only be mitigated by analysing results according to the groups into which the patients were randomized. Therefore, although the intention to treat approach may 'dilute' the result of a trial by including patients who did not actually receive active treatment, this approach ensures that the internal validity of any observed treatment difference is preserved.

Use of a prespecified analysis plan can prevent bias in the reporting of results of a clinical trial by avoiding undue emphasis of a data-derived subgroup[32]. Investigators often analyse data from clinical trials by subgroups within which the responses to treatment are expected to be fairly similar, but between which the responses are expected to differ. However this approach is seriously flawed. A key principle for interpretation of subgroup results is that quantitative interactions (differences in degree) are much more likely than qualitative interactions (differences in kind). Quantitative interactions are likely to be truly present whether or not they are apparent, whereas apparent qualitative interactions should generally be disbelieved as they have usually not been replicated consistently. Therefore, the overall trial result is usually a better guide to the direction of effect in subgroups than the apparent effect observed within a subgroup. Failure to specify prior hypotheses, to account for multiple comparisons, or to correct P values for multiple comparisons increases the chance of finding spurious subgroup effects. Conversely, inadequate sample size, classification of patients into the wrong subgroup, and low power of tests of interaction limit the power of a study to detect true subgroup effects. To avoid these potential pitfalls, the architecture of the entire set of subgroups within a trial should be carefully examined, similar subgroups should be analysed across independent trials, and results of subgroup analyses should be interpreted in the context of known biologic mechanisms and patient prognosis[32].

Decisions regarding therapies should be based on the totality of information (from all randomized trials)

In order to avoid selective biases and to minimize random errors, inference about the effects of treatment should not be based on a selective subset of the available trial results, but on a systematic overview of the totality of the evidence from all the relevant unconfounded randomized trials[2]. This fundamental principle is often overlooked, especially by clinical trialists who want to believe that their trial results warrant emphasis over and above other trials in area, and can result in bias[2,14,11]. The play of chance is more likely to influence the results of individual trials which can, in turn, determine which trials are emphasized and which trials are ignored. Therefore, proper assessment of a therapeutic issue must involve a compilation of all the available evidence, rather than a potentially biased subset of trials.

What are the expected findings in a trial of a new antiplatelet agent in a patient with vascular disease and what are their implications for the design of new trials?

In designing a clinical trial of an antiplatelet agent in patients with vascular disease, there are several key expectations that can guide the approach to the sound design of clinical trials.

Moderate (not large) treatment effects are likely to be observed

Moderate treatment benefits

A fundamental feature of a randomized trial addressing an important question in a common disease is that moderate treatment benefits (i.e. risk reductions of 15–30%) rather than large treatment effects (i.e. greater than 50%) are likely to be observed and that such moderate differences are clinically worthwhile[1]. Overwhelming treatment effects (e.g. defibrillation for ventricular fibrillation) may be apparent even in the absence of randomized trials, although, even in such cases, the magnitude of benefit may be more reliably and persuasively established by a randomized controlled trial. However, the impact of the vast majority of new treatments is 'reasonably uncertain', and the effects of these new treatments are likely to be small, moderate or neutral, rather than large. Nevertheless, small or moderate risk reductions may be worth detecting, particularly if the disease entity is common and if the outcome measures are clinically important such as mortality or major morbidity (such as myocardial infarction or stroke). For example, the use of aspirin in patients with acute myocardial infarction reduced the risk of vascular death by 23% in the ISIS-2 trial, a treatment effect which is 'only moderate'. This 23% reduction in mortality represents the avoidance of about 25 deaths per 1000 patients treated with aspirin for 1 month. Since aspirin is a simple, inexpensive and relatively non-toxic treatment, this moderate treatment effect translates in to tens of thousands of lives potentially saved per year, if aspirin is used widely. Tangible gains from small or moderate treatment effects of important outcomes in common

Cambridge Books Online © Cambridge University Press, 2009

disorders have had, and will continue to have, profound effects on global health and the modern practice of medicine. These treatments may exceed the numbers of lives that could hypothetically be saved by a simple cure for all patients with a rare disease, such as acute myeloid leukemia[1].

Minimizing random error and ensuring study power

Having established that biases should be avoided and that moderate treatment effects are generally worth detecting, the next step is to ensure that these effects can be detected reliably. The reliable detection of moderate treatment effects in randomized trials requires maintenance of statistical power by ensuring that a sufficient number of primary outcome events are collected. In general, this requires a study of sufficient size to accrue between 1000 and 2000 primary outcome events. Achieving this number of outcome events should be the starting point in the design of any major phase-3 randomized trial (this does not apply to phase-2 trials) of antiplatelet therapy in cardiovascular disease, because it has important implications on not only sample size but also inclusion criteria (and hence baseline event rate) and choice of primary outcome (single vs. composite). It is important that Steering Committees monitor the overall event rates during the course of a trial (remaining blinded to treatment allocation) to ensure that the projected event rates are actually being attained. If not, then it may be reasonable to either increase the sample size, to alter the entry criteria to focus on higher risk patients or increase the duration of treatment to ensure that a minimum number of events accrue during treatment (Table 68.2).

Two other pitfalls which may lead to an underpowered trial are the overestimation of the expected treatment effect of a particular agent or overestimation of the control event rate. In some instances, it may not be possible for a clinical trial to enroll sufficient numbers of patients to maintain adequate power to detect a given outcome, either for practical or economic reasons. Two commonly adopted methods of dealing with this problem are to use composite outcomes or to amend the inclusion criteria to focus on higher risk patients. The basic tenet of both these manoeuvres is to ensure that a minimum number of outcome events occur to maintain study power (Table 68.3).

Active vs. control randomized trials and trials comparing various active treatments (Table 68.4)

Treatment effects in clinical trials will vary, depending on whether the experimental agent is compared with control

Table 68.2. Key issues (based upon previous clinical trial results) which should affect the design of trial of antiplatelet agents in vascular disease

Key issue	Aspects of the clinical trial which will be affected
Expect moderate (not large) treatment effects	Ensure study power is maintained by recruiting large numbers of patients
Minimizing competing risks	Choice of endpoints, disease-specific vs. all-cause mortality
True unanticipated qualitative treatment effects are extremely rare	Use broad inclusion criteria ('uncertainty principle'); minimize exclusions
Treatment benefit will be greatest during treatment	Measure outcomes at or soon after end of treatment
Benefits will be clearer if treatment modifiable endpoints are chosen	Avoid including outcomes that are unlikely to be affected by treatment

Table 68.3. Potential strategies to maintain study power. In general, a phase III antiplatelet trial should record at least 1000 to 2000 primary outcome events in order to be adequately powered

Increase sample size
Alter entry criteria to focus on higher risk patients
Choose responsive and valid composite outcomes
Implement strategies to ensure optimal compliance of study medication

Table 68.4. Common pitfalls in the design of randomized trials

1	Overestimation of control event rates or treatment effects resulting in an underpowered trial
2	Failure to appreciate the difference in treatment effects between trials of active treatment vs. placebo and trials of active treatment vs active treatment
3	Entry criteria are too narrow, resulting in difficult recruitment and poor generalizabilty
4	Incomplete follow-up
5	Timing of primary outcome too long after end of active treatment

Cambridge Books Online © Cambridge University Press, 2009

(or placebo) or whether it is compared to another active agent. While moderate treatment effects (e.g. up to 20–30% relative risk reduction) may be seen when an effective agent is compared to placebo, the difference between two effective agents is likely to be much smaller (i.e. about half, which translates into relative risk reductions of 10 to 15%). Therefore, trials comparing active drugs may need to be substantially larger than placebo-controlled trials and may require a three- to fourfold greater number of events. This issue is of particular relevance to trials of antiplatelet therapy, where at least in the case of atherosclerotic disease, new agents must be compared to aspirin, which is a well-established and effective therapy, rather than placebo. Sometimes, a new agent may be compared in addition to aspirin vs. aspirin alone (plus placebo). Larger treatment benefits might be expected in this type of trial compared with a trial of the new agent alone vs. aspirin, assuming there is a sound rationale for dual antiplatelet therapy. For example, the CURE trial compared clopidogrel plus aspirin to placebo plus aspirin[15]. In this trial, the combination of aspirin plus clopidogrel compared with aspirin alone might be expected to yield greater treatment effects than simply comparing clopidogrel to aspirin, as in the CAPRIE study[16]. In the CAPRIE study the comparison of clopidogrel to aspirin resulted in a very modest 9% reduction in vascular events. By contrast, in a meta-analysis of the trials of clopidogrel vs. placebo in addition to aspirin (in patients receiving an intracoronary stent), risk reductions of 26–48% were observed[15]. Similarly in the CURE trial there was a 20% risk reduction in CV death, MI and stroke in favour of clopidogrel compared with placebo in patients receiving aspirin[37].

Adherence to randomized treatments

Lack of adherence to study treatments is one of the most important threats to maintaining power in a randomized trial. Adherence may include both those patients who temporarily or permanently discontinue study medication ('drop-outs'); those patients who take the study medication, but incorrectly; and those patients who take open-label study drug ('drop-ins'). The effect of non-adherence is likely to be greater than loss of study power resulting from low event rates because the statistical power of a study is proportional to the square of the proportion of non-adherent patients, but only directly proportional to the event rate. For example, in a study originally designed to have 90% power under a given set of assumptions, a change in adherence to 70% would decrease power to approximately 50%. Thus the issue of non-adherence requires close monitoring during the course of a trial to ensure that statistical power is maintained.

Use of composite outcomes

The selection of appropriate measures to assess the effect of therapy is critically important in a randomized trial. In general, outcome measures should be objective, valid, reliable, sensitive to change and clinically relevant. Mortality fulfils most of these criteria and is commonly used as a primary outcome in major cardiovascular and cancer trials. However, in many other disease states, use of mortality as a primary outcome would require an impractical number of patients, because of its low incidence. Therefore, composite outcomes that include mortality as one component are often employed to ensure accrual of sufficient primary outcome events. Death or myocardial infarction is a commonly used composite primary outcome in cardiovascular trials because myocardial infarction fulfills almost all of the criteria mentioned above, although it does, under some circumstances, require clinical judgement to make the diagnosis. Stroke may also be a suitable component of a primary composite outcome in trials of antiplatelet therapy in cardiovascular medicine because patients with coronary artery disease also have cerebrovascular disease, and the antiplatelet agent may prevent cerebrovascular atherothrombotic events or cardioembolic events resulting in stroke as well. For example, in both the CURE[15,37] and the BRAVO[17] trials, in which an oral antiplatelet was given for a few months in aspirin-treated patients (clopidogrel in CURE and lotrifiban in BRAVO), the primary outcomes were death, MI or stroke (cardiovascular death in CURE and all-cause death in BRAVO).

Refractory angina is a significantly more subjective endpoint than either death or myocardial infarction, but is increasingly being used in clinical trials of antiplatelet or antithrombotic therapies in an attempt to reduce sample size and study costs. However, a review of several trials in this area demonstrates that there are widely differing definitions of refractory angina among the various trials. Generally, a definition of refractory angina consists of three essential components: (i) signs or symptoms of recurrent ischemia (or manifestations such as pulmonary edema) for a specified duration of time; (ii) electrocardiographic changes and (iii) the performance of an intervention prompted by this episode (such as cardiac catheterization, a revascularization procedure, thrombolytic therapy, etc.). Most trials require two of three criteria, although some trials require only one out of three criteria and others require all three. The objectivity of this endpoint will depend on how the individual components of the outcome are defined (i.e. duration of chest pain, degree of ECG change required, etc.) and the number of criteria in the

Cambridge Books Online © Cambridge University Press, 2009

definition. As is often the case, the primary endpoint, consisting of death, MI or refractory ischemia is almost invariably driven by the latter, most subjective component. Readers are therefore cautioned when interpreting the results of clinical trials which have refractory ischemia as a primary endpoint, especially when the trial therapies demonstrate no impact on death or myocardial infarction.

Unanticipated qualitative treatment effects are rare

A qualitative interaction is one in which the direction of treatment within subgroups of patients within a trial is in different directions. As alluded to previously, true qualitative interactions are exceedingly rare, a fact that has important implications for the selection of patients in a clinical trial[18]. First, it implies that entering a wide group of patients with the same disease would be an asset because direct evidence for benefit can be sought in such a heterogeneous population. This makes the study results more relevant to clinical practice. Secondly, selection of wide categories of patients with the same disease is unlikely to reduce the observed treatment effect to such a significant extent that it outweighs the benefits of greater recruitment and broader clinical relevance. Thirdly, since even apparently similar patients are likely to differ quantitatively in their response to treatment, precise characterization of patients is usually not worthwhile. This, in turn, implies that patients entering a trial need only be broadly characterized using those few baseline variables that are known to provide powerful prognostic information, those that might modify the effects of a particular treatment or those that may be part of the endpoint in some surrogate endpoint trials.

This has led to what has been termed as the 'uncertainty principle', which states that the sole eligibility criterion is that both patient and doctor should be substantially uncertain about the appropriateness for this particular patient for each of the trial treatments[19]. This principle has been used successfully to simplify and therefore facilitate recruitment into many trials, such as the ISIS studies.

A common pitfall in the design of clinical trials of antiplatelet therapy is that a homogeneous patient population should be studied in order for the trial to be valid[18]. Thus it is often the case in trials of antiplatelet therapy that the criteria for recruitment is exceedingly narrow, thus severely limiting the generalizability of the study's findings and posing unnecessary hurdles to smooth and prompt patient recruitment. For example, there is good evidence that abciximab reduces major cardiovascular events in a wide variety of patients undergoing PCI, an effect which is sustained long term. The initial trial which established the benefits of abciximab in the context of PCI was the EPIC trial, which was performed only in patients undergoing PTCA at high risk of abrupt closure, a fraction of the total number of patients undergoing PTCA[20]. It was only subsequently in the EPILOG trial where the inclusion criteria broadened to include almost any patient undergoing PTCA (both elective and urgent), that it became apparent that the treatment benefit was present in all patients undergoing PTCA, not only high-risk patients[21]. These broad inclusion criteria were also adopted in the EPISTENT trial, which was consistent in demonstrating a benefit of abciximab in both elective and urgent PTCA in patients undergoing stenting[22].

CAPRIE is another example of a randomized trial of antiplatelet therapy with wide inclusion criteria[16]. This trial included patients with coronary artery disease, cerebrovascular disease and peripheral vascular disease because all these entities are due to a common pathophysiological mechanism. Similarly, the BRAVO trial of lotrafiban versus aspirin included patients with cardiovascular, cerebrovascular and peripheral vascular disease[17]. Meanwhile, results of the Antiplatelet Trialists' Collaboration meta-analysis clearly demonstrated consistent treatment effects across all these disease entities[23]. This is not surprising considering that occlusive vascular disease (atherothrombosis) is the common underlying pathophysiological mechanism for all these disease entities.

Treatment effect will be greatest during the treatment period

One basic principle of clinical trials of antiplatelet therapy is that the greatest treatment benefit will be observed during active treatment. Therefore, the optimum time to assess both efficacy and safety is either at the end of treatment or shortly thereafter. Following treatment cessation, loss of benefit may occur in the setting of clinical rebound, but there is unlikely to be any added benefit. Consequently, the time-to-event curves are likely to be parallel, reflecting maintenance of the absolute treatment benefit and apparent diminution of the relative treatment benefit. Lack of statistically significant treatment effect during long-term follow-up may be mistakenly interpreted as a 'loss of benefit'. However, careful analysis of the data will often reveal that no loss of benefit occurs, but rather, no added benefit occurs once treatment is stopped. Table 68.5 illustrates this pattern in five large randomized trials of antithrombotic therapy.

Cambridge Books Online © Cambridge University Press, 2009

Table 68.5. Efficacy results of five clinical trials during or just after the end of treatment and at 30 days. The lower treatment effect at 30 days does not represent a 'loss of benefit', but rather that no added benefit accrues once treatment is stopped

			Event rates and relative risk reduction (Death or myocardial infarction)	
	Antiplatelet/Anticoagulant	Duration of treatment	During or just after treatment (%)	30 days (%)
PRISM PLUS[27]	Tirofiban vs. placebo[a,b]	72 hours	0.6 vs. 2.0; RRR 70	6.8 vs. 11.7; RRR 42
PURSUIT[30]	Eptifibatide vs. placebo[a]	72 hours	7.6 vs. 9.1; RRR 16.4	14.2 vs. 15.7; RRR 9.6
FRISC[35]	Dalteparin vs. placebo	6 days acute phase	1.8 vs. 4.8; RRR 67	14.0 vs. 15.5; RRR 10
PRISM[28]	Tirofiban vs. UFH	48 hours	1.2 vs. 1.6; RRR24	5.8 vs. 7.1; RRR 20
GUSTO IIb[36]	Desirudin vs. UFH	72 hours	2.3 vs. 3.1; RRR 17	8.9 vs. 9.8; RRR 11
OASIS-2[26]	Lepirudin vs. UFH	72 hours	2.0 vs. 2.6; RRR 24	6.8 vs. 7.7; RRR 13

Notes:
[a] Both groups received unfractionated heparin. [b] Only non-discontinued arms.

The 'durability of effect' is also relevant and, for that reason, a 30-day endpoint is often chosen for treatment durations which are substantially shorter. However, it is less important if the results are statistically significant at this late time point. What matters more is whether the original difference (in absolute terms) is maintained during longer-term follow-up.

Confirming the maintenance of early benefit during longer-term follow-up is a complex issue. If, truly, there is no loss or gain of the original benefit during treatment, the risk reduction will appear to be maintained only some of the time but the absolute risk reduction will be constant. In other situations, the play of chance may exaggerate or reduce the observed treatment effect over time. For example, in the EPIC trial, the absolute treatment benefit of abciximab appeared to increase between 30 days and 6 months[20], but the reverse was observed in the CAPTURE trial[24]. Meanwhile, in the EPILOG study a consistent treatment benefit was maintained[34]. The most likely explanation for these apparently disparate results is the play of chance; in all likelihood, the true treatment benefit was maintained in all three cases.

The effect of cardiac interventions (when performed during study drug administration) in a trial of antiplatelet therapy will increase the event rate and the treatment effect

There are two main effects of cardiac interventions on the results of randomized trials of antiplatelet therapy[25]. First, interventions increase both the control and the treatment event rates. Secondly, interventions tend to exaggerate the treatment effect of the antiplatelet agent. These phenomena are demonstrated in Fig. 68.1 and Table 68.6. Therefore, in a clinical trial of a new antiplatelet agent in acute coronary syndrome, the performance of interventions as per usual clinical practice, will usually generate treatment-modifiable events. Suppressing interventions early on in these trials, may result in a reduced event rate. For example, in the OASIS-2 study[26], investigators were requested to delay interventions until after the 72-hour treatment period. However, among the small number of patients who underwent early intervention, the treatment effect was large. Had more interventions been performed early in this trial, the event rate would likely have been higher and the treatment effect of hirudin increased.

Several unique issues relate to the use of invasive procedures in trials of antiplatelet therapy in cardiovascular medicine. For example, in the PRISM PLUS trial[27], over 80% of patients received an intervention raising the possibility that the study drug (tirofiban) is only useful in the setting when an ACS patient can be taken to the catheterization lab early. By contrast, the difference observed in the PRISM study[28] was modest and no differences were observed in the GUSTO IV study[29] – both studied patients in centres without immediate access to PTCA. In the PCI CURE Study[38], there was a 31% reduction in CV death or MI ($P = 0.002$) in the large number of patients undergoing PCI ($N = 2658$); however, there was also a clear benefit of clopidogrel in those patients not undergoing PCI (relative risk 0.83; $P = 0.004$).

Cambridge Books Online © Cambridge University Press, 2009

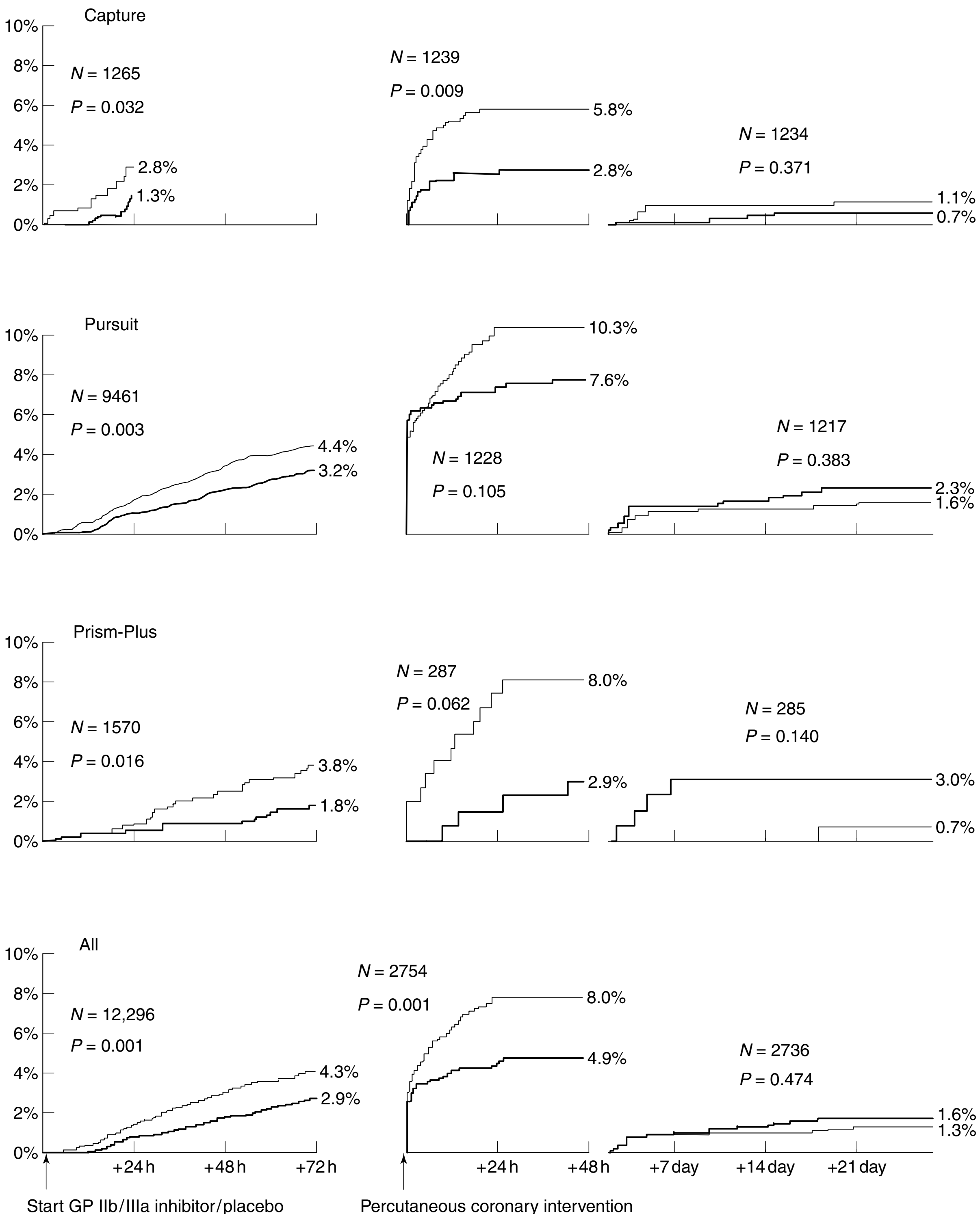


Fig. 68.1. Kaplan–Meier curves showing cumulative incidence of death or non-fatal myocardial (re)infarction in patients randomly assigned to GP IIb/IIIa inhibition (bold lines) or placebo. Adapted from ref.[25].

Cambridge Books Online © Cambridge University Press, 2009

Table 68.6. Impact of interventions on relative risk reduction in trials of antiplatelet agents

	Relative risk reduction	
Trial	Patients undergoing PCI(%)	Patients not undergoing PCI(%)
PURSUIT[30]	31	6
PARAGON B[31]	35	7
PRISM PLUS[27]	42	12
OASIS-2[26]	75	7
CURE[37,38]	31	17

In long-term trials, competing risks are important

Patients with cardiovascular disease are often an elderly cohort of patients who may have multiple overt or occult comorbidities. They are therefore prone to dying not only from cardiovascular disease, but also from other causes, such as malignancy. These other reasons for mortality are known as 'competing risks'. Because an antiplatelet agent would not be able to modify a non-cardiovascular death, these non-cardiovascular deaths would be expected to be balanced between treatment and control. This serves only to dilute out the treatment effect of the antiplatelet agent. It has been argued that distinguishing between cardiovascular and non-cardiovascular causes of death may be difficult and therefore this subjectivity diminishes the objectivity of mortality as an outcome. However, this is not true if, in a cardiovascular trial, one defines all deaths as cardiovascular, unless proven otherwise. Thus, the onus is upon the investigator to 'prove' that the death was not cardiovascular in nature. For example, a CT scan demonstrating a herniating brain tumour in a patient who died of coma might be ruled as non-cardiovascular, or a patient who develops acute leukemia, which is substantiated by pathological specimens or autopsy, might be ruled as non-cardiovascular. Deaths due to unknown causes, motor vehicle accidents, bleeding, etc. are all considered cardiovascular. When a cause of death is unknown in a trial of a patient who already has cardiovascular disease, 80–90% of the time, the death is due to cardiovascular causes. This minimizes any impact due to the subjectivity in classifying the cause of death. Others have argued that cardiovascular death fails to include those patients who may have a side effect from treatment, for example bleeding or severe bone marrow suppression. However, bleeding would be considered a cardiovascular death, as would any complication of a cardiovascular procedure, such as wound infection following CABG surgery. For example, there was a consistent reduction in CV death with clopidogrel (318 clopidogrel vs. 345 with placebo), with similar numbers of non-CV death (41 clopidogrel vs. 45 placebo)[37]. As for the rare instances where the drug of interest may have a deleterious effect on another system, separating deaths between cardiovascular and non-cardiovascular actually allows the investigator to study and report on these findings, which may otherwise have been overlooked if all deaths were grouped together.

References

1 Yusuf S. Randomised controlled trials in cardiovascular medicine: past achievements, future challenges. *BMJ* 1999; 319: 564–568.

2 Yusuf S, Collins R, Peto R. Why do we need some large, simple randomized trials? *Stat Med* 1984; 3: 409–420.

3 Peto R. Why do we need some systematic overviews of randomized trials? *Stat Med* 1987; 6: 233–240.

4 Peto R. Clinical trial methodology. *Biomedicine* 1978; 28: 24–36.

5 Hennekens CH. Issues in the design and conduct of clinical trials. *JNCI* 1984; 73: 1473–1476.

6 Hennekens CH, Buring JE. Methodologic considerations in the design and conduct of randomized trials: the US Physicians' health study. *Controlled Clinical Trials* 1989; 10: 142S-150S.

7 Prescott RJ, Counsell CE, Gillespie WJ et al. Factors that limit the quality, number and progress of randomized controlled trials. *Health Technol Assess* 1999; 3: 1–143.

8 Schultz KF, Chalmers I, Hayes RJ et al. Empirical evidence of bias. Dimensions of methodological quality associated with estimates of treatment effects in controlled trials. *JAMA* 1995; 273: 408–412.

9 Colditz GA, Miller JN, Mosteller F. How study design affects outcomes in comparisons of therapy. I: *Medical Stat Med* 1989; 8: 441–454.

10 Moher D, Pham B, Jones A et al. Does quality of reports of randomized trials affect estimates of intervention efficacy reported in meta-analyses? *Lancet* 1998; 352: 609–613.

11 Yusuf S. Obtaining medically meaningful answers from an overview of randomized clinical trials. *Stats in Med* 1987; 6: 281–286.

12 Sackett DL, Gent M. Controversy in counting and attributing events in clinical trials. *N Engl J Med* 1979; 301: 1410–1412.

13 Pocock SJ, Hughes MD, Lee RJ. Statistical problems in the reporting of clinical trials. *N Engl J Med* 1987; 317: 426–432.

14 Collins R, Gray R, Godwin J et al. Avoidance of large biases and large random errors in the assessment of moderate treatment effects: the need for systemic overviews. *Stat Med* 1987; 6: 245–250.

15 Mehta SR, Yusuf S on behalf of the CURE Steering Investigators. The Clopidogrel in Unstable angina to prevent Recurrent Events (CURE) trial programme. Rationale, design

Cambridge Books Online © Cambridge University Press, 2009

and baseline characteristics including a meta-analysis of the effects of thienopyridines in vascular disease. *Eur Heart J* 2000; 21: 2033–2041.

16 The CAPRIE Steering Committee. A randomised, blinded trial of clopidogrel versus aspirin in patients at risk of ischaemic events. *Lancet* 1996; 348: 1329–1339.

17 Topol EJ, Easton JD, Amarenco P et al. Design of the Blockade of the glycoprotein IIb/IIIa Receptor to Avoid Vascular Occlusion (BRAVO) trial. *Am Heart J* 2000; 139: 927–933.

18 Yusuf S, Held P, Teo KK et al. Selection of patients for randomized controlled trials: Implications of wide or narrow eligibility criteria. *Stats in Med* 1990; 9: 73–86.

19 Peto R, Collins R, Gray R. Large-scale randomized evidence: Large, simple trials and overviews of trials. *Ann NY Acad Sci* 1993; 703: 314–340.

20 The EPIC Investigators. Use of a monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high-risk coronary angioplasty. *N Engl J Med* 1994; 330: 956–961.

21 The EPILOG Investigators. Platelet glycoprotein IIb/IIIa receptor blockade and low-dose heparin during percutaneous coronary revascularization. *N Engl J Med* 1997; 336: 1689–1696.

22 Topol EJ, Mark DB, Lincoff AM et al. for the EPISTENT Investigators. Outcomes at 1 year and economic implications of platelet glycoprotein IIb/IIIa blockade in patients undergoing coronary stenting: results from a multicentre randomized trial. *Lancet* 1999; 354: 2019–2024.

23 Antiplatelet Trialists' Collaboration. Collaborative overview of randomized trials of antiplatelet therapy – I: Prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. *BMJ* 1994; 308: 81–106.

24 The CAPTURE Investigators. Randomised placebo-controlled trial of abciximab before and during coronary intervention in refractory unstable angina: the CAPTURE study. *Lancet* 1997; 349: 1429–1435.

25 Boersma E, Akkerhuis M, Theroux P et al. Platelet glycoprotein IIb/IIIa receptor inhibition in non-ST-elevation acute coronary syndromes. Early benefit during medical treatment only, with additional protection during percutaneous coronary intervention. *Circulation* 1999; 100: 2045–2048.

26 Organisation to Assess Strategies for Ischemic Syndromes (OASIS-2) Investigators. Effects of recombinant hirudin (lepirudin) compared with heparin on death, myocardial infarction, refractory angina, and revascularisation procedures in patients with acute myocardial ischaemia without ST elevation: a randomized trial. *Lancet* 1999; 353: 429–438.

27 The Platelet Receptor Inhibition in Ischemic Syndrome Management in Patients Limited by Unstable Signs and Symptoms (PRISM-PLUS) Study Investigators. Inhibition of the platelet glycoprotein IIb/IIIa receptor with tirofiban in unstable angina and non-q-wave myocardial infarction. *N Engl J Med* 1998; 338: 1488–1497.

28 The Platelet Receptor Inhibition in Ischemic Syndrome Management (PRISM) Study Investigators. A comparison of aspirin plus tirofiban with aspirin plus heparin for unstable angina. *N Engl J Med* 1998; 338: 1498–1505.

29 GUSTO-IV Investigators. Presented at the annual scientific sessions of European College of Cardiology, Amsterdam, The Netherlands August 2000.

30 The PURSUIT Trial Investigators. Inhibition of platelet glycoprotein IIb/IIIa with eptifibatide in patients with acute coronary syndromes. *N Engl J Med* 1998; 339: 436–443.

31 The PARAGON Investigators. International, randomized, controlled trial of lamifiban (a platelet glycoprotein IIb/IIIa inhibitor), heparin, or both in unstable angina. *Circulation* 1998; 97: 2386–2395.

32 Yusuf S, Wittes J, Probstfield J et al. Analysis and interpretation of treatment effects in subgroups of patients in randomized clinical trials. *JAMA* 1991; 266: 93–98.

33 ISIS-2 (Second International Study of Infarct Survival) Collaborative Group. Randomised trial of intravenous streptokinase, oral aspirin, both, or neither among 17,187 cases of suspected acute myocardial infarction: ISIS-2. *Lancet* 1988; i: 349–360.

34 Lincoff AM, Tcheng JE, Califf RM et al. for the EPILOG Investigators. Sustained suppression of ischemic complications of coronary intervention by platelet GP IIb/IIIa blockade with abciximab. One-year outcome in the EPILOG Trial. *Circulation* 1999; 99: 1951–1958.

35 Fragmin during Instability in Coronary Artery Disease (FRISC) study group. Low-molecular-weight heparin during instability in coronary artery disease. *Lancet* 1996; 347: 561–568.

36 The Global Use of Strategies to Open Occluded Coronary Arteries (GUSTO) IIB Investigators. A comparison of recombinant hirudin with heparin for the treatment of acute coronary syndromes. *N Engl J Med* 1996; 335: 775–782.

37 Yusuf S, Zhaof F, Mehta SR, Chrolavidus S, Tognoni G, Fox KAA for the CURE Investigators. EVects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. *N Engl J Med* 2001; 345: 494–502.

38 Mehta SR, Yusuf S, Peters RJ et al. For the CURE Investigators. Effects of pretreatment with clopidogrel and aspirin followed by long-term therapy in patients undergoing percutaneous coronary intervention: the PCI–CURE Study. *Lancet* 2001; 358: 527–533.

Cambridge Books Online © Cambridge University Press, 2009

69

Antiplatelet therapy in cardiology

Herbert D. Aronow and Eric J. Topol

Department of Cardiovascular Medicine, The Cleveland Clinic, Ohio, USA

Introduction

Antiplatelet therapy has revolutionized the care of patients with cardiovascular disease. It is frequently utilized in patients with heart failure[1,2], valvular heart disease[3–8] and rhythm disturbances[9,10]. However, its greatest impact has been in the prevention and treatment of coronary artery disease (CAD). CAD is presently the leading cause of death[11], and fifth leading cause of disability[12] worldwide and is projected to be the number one cause of death and disability in the world by the year 2020[13]. Given its considerable contribution to the global burden of disease, and the substantial reductions antiplatelet therapy has made on its attendant morbidity and mortality, this chapter will focus on the role of antiplatelet therapy in CAD.

The platelet plays a pivotal role in the development and progression of stable CAD as well as in the pathogenesis of its unstable clinical presentations (i.e. unstable angina, non-ST elevation myocardial infarction (MI) and ST elevation MI)[14,15]. While the pathways of platelet activation are manifold[16], relatively few have been successfully exploited for the prevention and treatment of CAD (Fig. 69.1). This chapter will review the current evidence base for three types of antiplatelet agents: aspirin, adenosine diphosphate (ADP) receptor antagonists and platelet glycoprotein (GP) IIb/IIIa inhibitors. As detailed elsewhere in this book, aspirin inhibits platelet aggregation by interfering with the formation of cyclic prostanoids (thromboxane A_2, prostacyclin and other prostaglandins)[17], ticlopidine and clopidogrel do so by irreversible antagonism of ADP P2Y receptors[18], and GP IIb/IIIa inhibitors accomplish this via blockade of the integrin receptor $\alpha_{IIb}\beta_3$, the final common pathway of platelet aggregation[19].

Primary prevention

Aspirin

Four randomized trials, the British Doctors' Trial (BDT)[20], Physicians' Health Study (PHS)[21], Thrombosis Prevention Trial (TPT)[22] and Hypertension Optimal Treatment study (HOT)[23], have examined long-term aspirin therapy for the primary prevention of cardiovascular events (Table 69.1). Individually, three of these have demonstrated significant reductions in the incidence of MI and composite vascular events (coronary death or non-fatal MI in TPT; coronary death, non-fatal MI or non-fatal stroke in PHS and HOT)[21–23], while in the fourth[20], these reductions were non-significant.

In a meta-analysis of these four trials, aspirin's effect on vascular death was null (RR 1.01, 95% CI 0.88–1.16). However, aspirin therapy was associated with a significant reduction in vascular events (defined as cardiac death, non-fatal MI, or fatal or non-fatal stroke) (RR 0.87, 95% CI 0.81–0.95), a difference largely driven by reductions in non-fatal MI (RR 0.68, 95% CI 0.59–0.79). Nevertheless, its clinical benefits were offset by a significant increase in the risk of hemorrhagic stroke (RR 1.69, 95% CI 1.06–2.69). On balance, treating 10 000 patients with aspirin therapy for the primary prevention of cardiovascular events would prevent 67 MIs while causing an excess of 11 hemorrhagic strokes[24].

ADP receptor antagonists and GP IIb/IIIa inhibitors

To date, there have been no published studies of ADP or GP IIb/IIIa receptor antagonists for the primary prevention of cardiovascular disease.

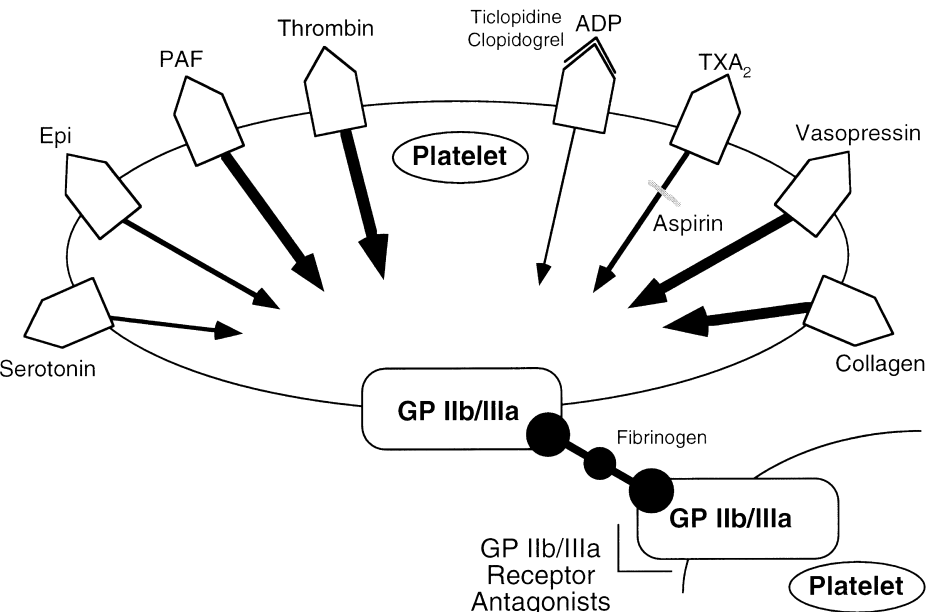


Fig. 69.1. Pathways of platelet activation and their pharmacologic inhibition. Epi = epinephrine; PAF = platelet activating factor; ADP = adenosine diphosphate; TXA2 = thromboxane; GP IIb/IIIa = platelet glycoprotein IIb/IIIa receptor. From[16]. Pending author's permission.

Secondary prevention

Aspirin

Six large randomized trials of aspirin alone for secondary prevention after acute MI have been published[25–30]. Aspirin was associated with a non-significant trend toward reduced total mortality excepting one[25], in which it was non-significantly increased. Significant benefits associated with aspirin therapy in individual trials include, decreased total mortality in patients enrolled within 6 months of an MI[28], decreased coronary mortality among men[30], and significantly reduced reinfarction or readmission for MI[25,29]. The results from these six trials were combined with those from 40 other smaller trials of aspirin monotherapy in a meta-analysis of more than 45 000 patients at high risk for suffering recurrent vascular events (defined as vascular death, non-fatal MI or non-fatal stroke). The pooled risk reduction afforded by aspirin monotherapy was highly significant (OR 0.75, 95% CI 0.70–0.79)[31].

ADP receptor antagonists

Two trials have assessed the efficacy of ADP receptor antagonism for the secondary prevention of cardiovascular disease, the Swedish Ticlopidine Multicentre Study (STIMS) and the Clopidogrel vs. Aspirin in Patients at Risk for Ischemic Events (CAPRIE) trial.

Ticlopidine

STIMS randomized 687 patients with a history of peripheral vascular disease (PVD), to ticlopidine or placebo and followed them for a mean of 5.9 years[32]. Ticlopidine was associated with a non-significant reduction in the primary composite endpoint of MI, stroke or TIA, (25.7% vs. 29.0%, $P = 0.24$), and a significant reduction in mortality (18.5% vs. 26.1%, $P = 0.015$), a difference primarily explained by reduced death from CAD.

Clopidogrel

CAPRIE randomized 19 185 patients with a recent acute MI, recent stroke or PVD to daily clopidogrel or aspirin for a mean of 1.9 years. The average yearly rate of ischemic stroke, MI or vascular death, the study's primary composite endpoint, was significantly reduced among patients in the clopidogrel group (5.32% vs. 5.83%, $P = 0.043$)[33]. A posthoc analysis revealed that clopidogrel was also associated with a significant reduction in MI (RR 0.81, 95% CI 0.69–0.95)[34]. The ongoing Clopidogrel for Reduction of Events During extended Observation (**CREDO**) trial, a study of patients with CAD undergoing percutaneous coronary interventions (PCI), is assessing whether clopidogrel

Cambridge Books Online © Cambridge University Press, 2009

Table 69.1. Primary prevention trials of aspirin therapy

	British Doctors' Trial (BDT)	Physicians' Health Study (PHS)	Thrombosis Prevention Trial (TPT)	Hypertension Optimal Treatment Study (HOT)
Period of enrolment	11/78–11/84	NA	1989–1992	10/92–04/94
Study size (n)	5139	22071	5085	18790
Population	Low-risk British male physicians	US male physicians	British men at high risk for ischemic heart disease	Men and women with diastolic hypertension (84% without coronary disease)
Treatment group/s	Aspirin 500 mg daily (n=3429)	Aspirin 325 mg every other day (n=11037)	Aspirin 75 mg controlled-release daily (n=2545)	Aspirin 75 mg daily (n=9399)
Control group	Avoidance (n=1710)	Placebo (n=11034)	Placebo (n=2540)	Placebo (9391)
Endpoint analysis	6 years (mean)	5 years (mean)	7 years (median)	4 years (mean)
Primary endpoint	Vascular mortality	Cardiovascular mortality	Coronary death, fatal or non-fatal MI	Cardiovascular death, non-fatal MI or non-fatal stroke
Primary endpoint[a]	0.94 [NA], P=NS	0.96 [0.60–1.54], P=0.87	0.79 [0.65–0.96], $P<0.05$	0.85 [0.73–0.99], P=0.03
Death (all-cause)[a]	0.90 [NA], P=NS	0.96 [0.80–1.14], P=0.87	0.83 [0.70–0.99], $P<0.05$	0.93 [0.79–1.09], P=0.36
Non-fatal MI[a]	0.98 [NA], P=NS	0.59 [0.47–0.74], $P<0.001$	0.90 [0.71–1.15], P=NS	0.64 [0.49–0.85], P=0.002[c]
Non-fatal stroke[a]	1.14 [NA], P=NS	1.20 [0.91–1.59], P=0.20	0.78 [NA], P=NS	0.98 [0.78–1.24], P=NS[c]
Bleeding[ab]	1.43 [NA], P=NS	1.32 [1.25–1.40], $P<0.001$	1.21 [NA], $P<0.001$	1.8 [NA], $P<0.001$

Notes:
MI = myocardial infarction; NA = not available; NS = non-signficant.
[a] RR [95% CI] for aspirin therapy vs. control.
[b] Bleeding defined as non-cerebral in BDT, any in PHS, minor in TPT and major in HOT.
[c] Includes fatal and non-fatal events (separate data on non-fatal events not published).

and aspirin in combination are superior to aspirin alone for preventing late ischemic events.

GP IIb/IIIa inhibitors

Intravenous GP IIb/IIIa inhibitors reduce ischemic complications in patients undergoing PCI and in those with acute coronary syndromes (ACS) (see below). Given that increased levels of platelet activation persist for some time after an ACS[35], a number of studies have examined whether prolonged treatment with oral GP IIb/IIIa inhibitors might offer additional protection after such an event (Table 69.2). Despite promising results in Phase II studies of sibrafiban[36] orbofiban[37], and xemilofiban[38], four Phase III clinical trials evaluating these three agents have failed to demonstrate clinical benefits (Table 69.2). Mortality was increased significantly in the second Sibrafiban vs. aspirin to Yield Maximum Protection from ischemic Heart events post-acute cOroNary sYndromes (2nd SYMPHONY)[39], Orbofiban in Patients with Unstable coronary Syndromes (OPUS-TIMI 16)[37] and Evaluation of oral Xemilofiban in ControllIng Thrombotic Events (EXCITE)[40] trials, and non-significantly increased in the SYMPHONY[41,42] trial. Significant increases in the composite endpoint of death or non-fatal MI were also noted in the second SYMPHONY trial, while non-significant increases in this endpoint were observed in the remaining three. Bleeding was significantly increased in all four of these studies. When the results of these four trials were meta-analysed, the increase in mortality associated with use of an oral GP IIb/IIIa inhibitor was highly significant (OR 1.37, 95% CI 1.13–1.66, P= 0.002)[43].

Despite these discouraging results, clinical outcomes from one Phase II study appear promising. The Fibrinogen Receptor Occupancy Study Trial (FROST) randomized 513 patients with unstable angina to low, intermediate or high doses of the oral GP IIb/IIIa receptor blocker lefradafiban. The incidence of the composite endpoint, death, MI, PCI or coronary artery bypass grafting (CABG) was lower in the intermediate-dose than placebo arm at 30 days[44], and this benefit persisted at 6 months (38.5% vs. 56.9%)[45]; the high-dose arm was terminated early due to unacceptably high

Cambridge Books Online © Cambridge University Press, 2009

Table 69.2. Secondary prevention trials of oral platelet glycoprotein IIb/IIIa inhibitors

	SYMPHONY	2nd SYMPHONY	OPUS TIMI-16	EXCITE
Period of enrolment	08/97–10/98	11/98–08/99	10/97–11/98	06/97–04/98
Agent	Sibrafiban	Sibrafiban	Orbofiban	Xemilofiban
Study size (n)	9233	6671	10 288	7232
Population	Within 7 days of an ACS and clinically stable × 12 hours	Within 7 days of an ACS and clinically stable × 12 hours	Within 72 hours of an ACS	30–90 minutes before percutaneous coronary intervention
Treatment group/s	(1) Low dose sibrafiban: 3.0–4.5 mg twice daily (weight- and Cr-adjusted) × 90 days, targeted to achieve 50% inhibition of platelet aggregation (n = 3105). (2) High-dose sibrafiban: 3.0–6.0 mg twice daily × 90 days (adjusted as above) targeted to achieve 80% inhibition of platelet aggregation (n = 3039).	(1) Low dose sibrafiban: 3.0–4.5 mg twice daily (weight- and Cr-adjusted) × 90 days, targeted to achieve 50% inhibition of platelet aggregation; aspirin 325 mg daily (n = 2232). (2) High-dose sibrafiban: 3.0–6.0 mg twice daily × 90 days (adjusted as above) targeted to achieve 80% inhibition of platelet aggregation (n = 2174)	(1) Low dose orbofiban: 50 mg twice daily, targeted to achieve 45–65% inhibition of platelet aggregation; aspirin 150–162 mg daily (n = 3537). (2) High-dose orbofiban: 50 mg twice daily for 30 days, then 30 mg twice daily, targeted to achieve 55–80% inhibition of platelet aggregation; aspirin 150–162 mg daily (n = 3330)	(1) Low-dose xemilofiban: 20 mg pre-procedure, then 10 mg three times daily × 6 months, targeted to achieve 40–70% inhibition of platelet aggregation; aspirin 80–325 mg daily (n = 2414). (2) High-dose xemilofiban: 20 mg pre-procedure and then three times daily, targeted to achieve 60–90% inhibition of platelet aggregation; aspirin 80–325 mg daily (n = 2400).
Control group	Aspirin 80 mg twice daily (n = 3089)	Aspirin 325 mg daily (n = 2231)	Placebo; aspirin 150–162 mg daily (n = 3421)	Placebo; aspirin 80–325 mg daily (n = 2418)
Endpoint analysis	90 days	90 days	30 days	6 months
Primary endpoint	All-cause mortality, non-fatal MI or severe recurrent ischemia	All-cause mortality, non-fatal MI or severe recurrent ischemia	Death, MI recurrent ischemia or stroke	Death, non-fatal MI or urgent revascularization

	SYMPHONY				2nd SYMPHONY				OPUS TIMI-16			EXCITE				
Outcome analysis	Aspirin	Low-dose	High-dose	P value	Aspirin	Low-dose	High-dose	P value	Placebo	Any dose	P value	Placebo	Low-dose	P value	High-dose	P value
Primary endpoint (%)	9.8	10.1	10.1	NS	9.3	9.2	10.5	NS	10.8	9.9	0.12	13.5	13.9	0.82	12.7	0.36
Death (%)	1.8	2.0	2.0	NS	1.3	1.7	2.4	<0.05	1.4	2.0[c]	0.02	1.0	1.7	0.04	1.1	0.68
Non-fatal MI (%)	5.6	5.8	6.5	NS	5.3	5.3	6.9	<0.05	2.9	2.8	0.89	8.4	8.1	0.56	7.5	0.23
Death or non-fatal MI (%)	7.0	7.4	7.9	NS	6.1	6.8	8.6	<0.05	3.9	4.4	0.31	8.9	9.2	0.87	8.2	0.36
Major bleeding[a] (%)	3.9	5.2	5.7	<0.05	4.0	5.7	4.6	<0.05	1.4	2.4 /2.6[b]	<0.001	1.8	5.1	<0.001	7.1	<0.001

Notes:

MI = myocardial infarction; ACS = acute coronary syndrome; Cr = creatinine; NS = non-significant.

[a] Major bleeding is defined as intracranial hemorrhage or fall in hemoglobin ≥5 g/dl for SYMPHONY and 2nd SYMPHONY; and, intracranial hemorrhage, fall in hematocrit of 15%, need for blood transfusion or causing hemodynamic compromise in EXCITE and OPUS-TIMI 16.

[b] low-dose/high-dose arm.

Cambridge Books Online © Cambridge University Press, 2009

bleeding rates. Further analysis revealed an inverse relationship between fibrinogen receptor occupancy and the clinical event rate[46]. Whether more individualized dosing of existing agents based on fibrinogen receptor occupancy will lead to improved clinical outcomes remains to be seen. Regardless, a number of promising new agents are under development or being assessed in ongoing or recently completed clinical trials, including fradafiban/lefradafiban[47–49], roxifiban[50,51], lotrafiban[52,53] and chromofiban.

Non-ST elevation acute coronary syndromes

Aspirin

The efficacy of aspirin for treating unstable angina and non-ST elevation MI (i.e. non-ST ACS) is well established. Four trials have demonstrated that aspirin reduces ischemic events in patients with non-ST elevation ACS (Table 69.3). In the Veterans Administration (VA) Cooperative Study[54], a trial by Theroux et al.[55], the Research Group on Instability in Coronary artery disease (RISC) study[56,57], and the Canadian Multicenter Trial[58], aspirin significantly reduced rates of the composite endpoint, death or non-fatal MI (in the latter trial, this association was only significant in the on-treatment analysis). In two of these, the VA Cooperative Study and Canadian Multicenter Trial, aspirin significantly reduced mortality rates at 1–2 years of follow-up, whereas in RISC and the study by Theroux et al., there were non-significant reductions in this endpoint among aspirin-treated patients. Finally, in the study by Theroux et al., the composite endpoint, death, non-fatal MI or refractory ischemia was significantly reduced with aspirin therapy. Importantly, bleeding occurred no more frequently in aspirin- than placebo-treated patients in any of these trials. In sum, these studies demonstrate that aspirin offers significant protection against death, MI and refractory ischemia without an attendant increase in hemorrhagic risk when used for non-ST elevation ACS.

ADP receptor antagonists

Ticlopidine

Only one trial has examined the effect of ADP receptor antagonism for non-ST elevation ACS. Studio della Ticlopidine nell' Angina Instabile (STAI) randomized 652 patients with unstable angina to 6 months of conventional therapy (i.e. beta blockers, calcium channel blockers or nitrates, but not aspirin) alone or in combination with ticlopidine 250 mg twice daily. The addition of ticlopidine significantly reduced the incidence of vascular death or MI (7.3% vs. 13.6%, OR 0.50, 95% CI 0.30–0.84; $P=0.009$)[59,60].

Clopidogrel

While the efficacy of aspirin and ticlopidine for the treatment of non-ST elevation ACS has been clearly demonstrated, these agents have not been compared in a head-to-head fashion, nor have they been evaluated in combination in this setting. The ongoing Clopidogrel in Unstable angina to prevent Recurrent ischemic Events (CURE) trial is randomizing 12 500 patients with unstable angina to aspirin plus placebo or aspirin plus clopidogrel to assess the incremental effect of ADP receptor antagonism on vascular death and non-fatal MI[61].

GP IIb/IIIa inhibitors

After preliminary clinical data suggested that the intravenous GP IIb/IIIa inhibitors tirofiban[62], lamifiban[63], eptifibatide[64] and abciximab[65–67] reduce the incidence of ischemic events in patients with non-ST elevation ACS, six phase III trials evaluating these agents were performed (Table 69.4).

Tirofiban

In the Platelet Receptor Inhibition in Ischemic Syndrome Management (PRISM) trial, tirofiban treatment was associated with a reduction in death, non-fatal MI or refractory ischemia at 48 hours, the study's primary endpoint[68]. This benefit attenuated thereafter, such that it was no longer statistically significant at 7 or 30 days. Nevertheless, when compared with heparin at 30 days, tirofiban reduced the incidence of death in the entire cohort. Furthermore, it reduced the composite of death, non-fatal MI or refractory ischemia among patients who underwent early PCI (21.6% vs. 27.3%, $P<0.05$) and reduced the risk of death (1.6% vs. 6.2%, $P=0.004$) and MI (2.7% vs. 6.8%, $P=0.01$) at 30 days among patients with an elevated baseline troponin level[69]. These benefits did not occur at the expense of increased bleeding risk. The Platelet Receptor Inhibition in Ischemic Syndrome Management in Patients Limited by Unstable Signs and Symptoms (PRISM–PLUS) study, randomized patients to one of three regimens: standard-dose tirofiban (as in PRISM), low-dose tirofiban with heparin or heparin alone[70]. The tirofiban-only arm was stopped prematurely given its higher incidence of death at seven days. Nevertheless, when compared with heparin alone, the tirofiban-heparin combination clearly reduced the composite primary endpoint of death, non-fatal MI, refractory

Cambridge Books Online © Cambridge University Press, 2009

Table 69.3. Trials of aspirin for the treatment of non-ST elevation acute coronary syndromes

	VA Cooperative	Canadian Multicenter	Theroux et al.	RISC
Period of enrolment	11/74–08/81	07/79–12/83	10/86–03/88	05/85–06/88
Study size (n)	1266	555	479	796
Population	Men with unstable angina in first 48 hours	Unstable angina admitted to a coronary care unit	Acute phase unstable angina	Men with unstable angina or non-Q wave MI in a coronary care unit
Treatment group/s	Aspirin 324 mg daily × 12 weeks (n = 625)	(1) Aspirin 325 mg 4 times daily (n = 139) (2) Sulfinpyrazone 200 mg 4 times daily (n = 140) (3) Aspirin 325 mg 4 times daily; sufinpyrazone 200 mg 4 times daily (n = 137)	(1) Aspirin 650 mg once, then 325 mg daily (n = 121) (2) Intravenous heparin (n = 118) (3) Aspirin 650 mg once, then 325 mg daily; heparin (n = 122)	(1) Aspirin 75 mg daily × up to 1 year (n = 399) (2) Intravenous heparin × 5 days (n = 388) (3) Aspirin 75 mg daily; heparin (n = 408)
Control group	Placebo (n = 641)	Placebo (n = 139)	Placebo (n = 118)	Aspirin placebo (n = 397); Heparin placebo (n = 388)
Endpoint analysis	12 weeks	Mean 18 months	6 days	3 months
Primary endpoint	All-cause mortality	Cardiac death or non-fatal MI	Death, MI or refractory ischemia	Death or non-fatal MI

Outcome analysis	VA Cooperative: Placebo	Aspirin	P value	Canadian Multicenter: No aspirin	Aspirin	P value	Theroux et al.: Placebo	Aspirin	P value	RISC: Aspirin placebo	Aspirin	P value
Primary endpoint (%)	3.3	1.6	0.054[a]	10.0	5.8	0.035	26.3	16.5	0.066	17.1	6.5	<0.001
Death (%)	3.3	1.6	0.054[a]	10.0	5.8	0.035	1.7	0	NS	2.5	1.5	NS
Non-fatal MI (%)	6.9	3.4	0.005	NA	NA	NA	11.9	3.3	0.012	NA	NA	NA
Death or non-fatal MI (%)	10.1	5.0[c]	0.001	14.7	10.5	0.072[b]	13.6	3.3	0.004[c]	17.1	6.5	<0.001

Notes:
MI = myocardial infarction; NA = not available; NS = non-significant.
[a] At 1 year, respective mortality in placebo and aspirin groups: 9.6% vs. 5.5%, $P = 0.008$.
[b] In an on-treatment analysis, death/non-fatal MI in aspirin vs. control: 17.6% vs. 9.6%, $P = 0.008$.
[c] Statistic not provided in study; calculated from data provided using Mantel–Haenszel chi-square test.

Cambridge Books Online © Cambridge University Press, 2009

ischemia or rehospitalization at both 30 days and 6 months and these benefits were only offset by a small increase in bleeding risk. Based on these results, heparin is now routinely administered with tirofiban in non-ST elevation ACS.

Lamifiban

The PARAGON-A trial randomized patients to either placebo with heparin, low-dose lamifiban with or without heparin or high-dose lamifiban with or without heparin[71]. At 30 days, the incidence of death or non-fatal MI, the study's primary endpoint, was not significantly different between groups. However, by six months, there was a statistically significant reduction in death or non-fatal MI among all patients treated with low-dose lamifiban when compared with placebo, irrespective of concomitant heparin administration. As expected, major or intermediate bleeding occurred more frequently in high- and low-dose groups than in placebo. In subsequent posthoc analyses of these data, those with intermediate serum lamifiban concentrations (i.e. 18–42 ng/ml) witnessed the greatest event reductions at 30 days and 6 months when compared with placebo and these reductions persisted at six months[72]. Accordingly, the PARAGON-B[73] trial evaluated the effect of treatment to intermediate serum lamifiban levels. This trial randomized patients to placebo or renal-dosed lamifiban. The primary efficacy endpoint, a composite of death, non-fatal MI or severe recurrent ischemia at 30 days was non-significantly reduced while bleeding complications were more frequent among lamifiban-treated patients. However, lamifiban significantly reduced the incidence of the primary endpoint at 30 days among patients who underwent early PCI (11.6% vs. 18.5%, $P<0.05$) and in those with a positive troponin level on admission (11.0% vs. 19.4%, $P=0.01$)[74], a finding similar to that seen in other trials of IIb/IIIa inhibitors for non-ST elevation ACS.

Eptifibatide

In the Platelet Glycoprotein IIb/IIIa in Unstable Angina: Receptor Suppression Using Integrilin Therapy (PURSUIT) trial, patients were randomized to eptifibatide or placebo for 72 hours or until hospital discharge, whichever occurred sooner. The primary endpoint, death or non-fatal MI, was significantly reduced in the eptifibatide group at 30 days and this benefit persisted at 6 months. Not surprisingly, bleeding was significantly more frequent among those treated with eptifibatide. In a substudy that included only US patients, eptifibatide significantly reduced the composite endpoint, death or non-fatal MI, at 30 days and 6 months, reductions that were greater in magnitude than those observed in non-US patients[75]. In contrast to patients who underwent early PCI in trials of tirofiban and lamifiban, the risk reduction attributable to eptifibatide was similar in patients who underwent early PCI and in those who were managed more conservatively[76].

Abciximab

Finally, the Global Use of Streptokinase or t-PA for Occluded Coronary Arteries–Acute Coronary Syndromes (GUSTO IV–ACS) trial randomized patients to abciximab for 24 or 48 hours, or to placebo. This trial revealed a trend toward increased ischemic events and significantly increased rates of bleeding among patients assigned to the 24– and 48-hour abciximab arms. Furthermore, no benefits were observed with abciximab in patients with positive troponin levels or with ST-depression on baseline ECG.

The trend towards harm associated with abciximab in GUSTO IV-ACS, especially during study drug administration, occurred despite the apparent benefits observed in patients with ACS who underwent PCI in earlier trials of abciximab[65–67] and in contrast to accumulating trial evidence supporting the use of tirofiban, eptifibatide and possibly lamifiban for ACS. Whether this lack of benefit was due to the selection of patients with a lower risk profile, the use of a suboptimal dosing strategy, the administration of abciximab in a non-PCI setting or to some other property of the agent itself will require further study. Recent data suggesting pro-inflammatory effects, such as the shedding of CD40 ligand receptors due to suboptimal GP IIb/IIIa receptor blockade, have also been cited as an explanation. Nevertheless, the ability of GP IIb/IIIa inhibitors to reduce ischemic events after a non-ST elevation ACS remains well established. In a meta-analysis of the PRISM, PRISM-PLUS, PARAGON A and B, PURSUIT and GUSTO IV–ACS trials, GP IIb/IIIa receptor blockade significantly reduced 30-day death or non-fatal MI (pooled OR 0.92, 95% CI 0.86–0.995, $P=0.037$; Breslow–Day test for heterogeneity: $P=0.34$)[77].

ST elevation acute coronary syndromes

Aspirin

Although one earlier randomized trial of 1682 patients had suggested that early aspirin therapy after an acute MI could reduce mortality[29], aspirin's overwhelming and unquestionable benefit in this setting was demonstrated in the landmark International Study of Infarct Survival (ISIS) – 2[78]. In that trial, 17 187 patients within 24 hours of a suspected acute MI were randomized to aspirin 162.5 mg daily

Cambridge Books Online © Cambridge University Press, 2009

Table 69.4. Trials of platelet glycoprotein inhibitors for the treatment of non-ST elevation acute coronary syndromes

	PRISM	PRISM-PLUS	PARAGON A
Period of enrolment	03/94–10/96	11/94–09/96	08/95–05/96
Agent	Tirofiban	Tirofiban	Lamifiban
Study size (*n*)	3232	1915	2282
Population	Non-ST elevation ACS	Non-ST elevation ACS	Non-ST elevation ACS
Treatment group/s	Tirofiban 0.6 μg/kg/min × 30 minutes, then 0.15 μg/kg/min × 47.5 hours (*n* = 1616)	(1) Tirofiban 0.6 μg/kg/min × 30 minutes, then 0.15 μg/kg/min × ≥47.5 hours (12–24 hours after PCI) (*n* = 345) (2) Tirofiban 0.4 μg/kg/min × 30 minutes, then 0.10 μg/kg/min × ≥47.5 hours (12–24 hours after PCI); heparin (*n* = 773)	(1) Low-dose lamifiban (300 μg bolus, 1 μg/min × 3–5 days) with heparin (*n* = 377) (2) Low-dose lamifiban (300 μg bolus, 1 μg/min × 3–5 days) without heparin (*n* = 378) (3) High-dose lamifiban (750 μg bolus, 5 μg/min × 3–5 days) with heparin (*n* = 373) (4) High-dose lamifiban (750 μg bolus, 5 μg/min × 3–5 days) without heparin (*n* = 396)
Control group	Placebo (*n* = 1616)	Placebo + heparin, (*n* = 797)	Placebo (*n* = 758)
Heparin dosing	Heparin 5000 U IV bolus, then 1000U/h infusion × 48 hours adjusted to aPTT 2x normal	Heparin 5000 U IV bolus, then 1000U/h infusion × 48 hours adjusted to aPTT 2x normal	One low-dose, one-high dose and placebo group received heparin 60 U/kg IV bolus and 12 U/kg/min infusion titrated to aPTT 60–85 s.
Aspirin dosing	Aspirin 300–325 mg before randomization and then × 48 hours, otherwise at physician's discretion	Aspirin 325 mg on day of randomization and daily thereafter	All received aspirin
Endpoint analysis	48 hours	7 days	30 days
Primary endpoint	Death, non-fatal MI or refractory ischemia	Death, non-fatal MI, refractory ischemia or rehospitalization for unstable angina	Death or non-fatal MI

Outcome analysis	Heparin	Tirofiban	*P* value	Placebo	Tirofiban	*P* value	Tirofiban + Heparin	*P* value	Plac	Low + Hep	Low − Hep	High + Hep	High − Hep	*P* value
Primary endpoint (%)	5.6	3.8	0.01	17.9	17.1	NA	12.9	0.004[d]	11.7	10.3	10.8	12.3	11.6	see below[f]
Death (%)	0.2	0.4	0.54[b]	1.9	4.6	0.012[c]	1.9	0.99	2.9	2.9	3.2	3.8	3.5	NA
Non-fatal MI (%)	1.4	0.9	0.19	7.0	7.0	NA	3.9	0.006	10.6	9.3	9.5	11.3	10.6	NA
Death or non-fatal MI (%)	1.6	1.2	0.38	8.3	10.4	NS	4.9	0.006	11.7	10.3	10.8	12.3	11.6	NA[g]
Major bleeding (%)[a]	0.4	0.4	NS	3.0	NA	NA	4.0	0.34[e]	0.8	0.5	0.8	2.4	1.3	NA[h]

Period of enrolment	11/95–01/97	02/98–06/99	NA
Agent	Eptifibatide	Lamifiban	Abciximab
Study size (*n*)	10948	5225	7800
Population	Non-ST elevation ACS	Non-ST elevation ACS	Non-ST elevation ACS. Excluded patients in whom PCI/CABG planned within next 30 days
Treatment group/s	Eptifibatide 180 μg IV bolus followed by 2.0 μg/kg/min infusion × 72 hours (*n* = 4722)	Lamifiban 500 μg bolus, then 1, 1.5 or 2.0 μg/min (adjusted for renal function to achieve serum concentration 20–40 ng/ml) for up to 72 hours (*n* = 2628)	(1) Abciximab 0.25 mg/kg bolus, then 0.125 ug/kg/min infusion × 24 hours (*n* = 2590). (2) Abciximab bolus and infusion × 48 hours (*n* = 2612).
Control group	Placebo bolus and infusion (*n* = 4739)	Placebo (*n* = 2597)	Placebo (2598)
Heparin dosing	5000 U IV bolus followed by 1000 U/hour infusion with adjustments to keep aPTT 50–70 s recommended	All patients received heparin 5000 U IV bolus and 1000 U/hour infusion (weight-adjusted if ≤80 kg) to keep aPTT 50–70 s (substitution with LMWH permitted)	Heparin 70 U IV bolus, then 10 U/kg/h infusion titrated to aPTT 50–70 s × 48 hours
Aspirin dosing	Aspirin 80–325 mg daily was encouraged	Aspirin 150–325 mg at randomization and daily thereafter	Aspirin 150–325 mg orally or 250–500 mg i.v. after randomization, then 75–325 mg orally each day for at least 1 month
Endpoint analysis	30 days	30 days	30 days
Primary endpoint	Death or non-fatal MI	Death, non-fatal MI or severe recurrent ischemia	Death or non-fatal MI

Outcome analysis	Placebo	Eptifibatide	*P* value	Placebo	Lamifiban	*P* value	Placebo	24-h	*P* value	48-h	*P* value
Primary endpoint (%)	15.7	14.2	0.04	12.8	11.8	0.33	8.0	8.2	*P* = NS	9.1	p = NS
Death (%)	3.7	3.5	0.53	3.3	2.9	0.49	3.9	3.4	*P* = NS	4.2	*P* = NS
Non-fatal MI (%)	13.5	12.6	0.14[i]	9.8	8.8	0.27	4.1	4.7	NA	4.9	NA
Death or non-fatal MI (%)	15.7	14.2	0.04[j]	11.5	10.6	0.32[k]	8.0	8.2	*P* = NS	9.1	p = NS
Major bleeding (%)[a]	9.1	10.6	<0.001	0.9	1.3	NS[l]	0.3	0.6	*P* = NS	1.0	<0.001

Notes:

MI = myocardial infarction; ACS = acute coronary syndrome; IV = intravenous; aPTT = activated partial thromboplastin time; NS = non-significant; NA = not available; s = seconds, LMWH = low molecular weight heparin; hep = heparin.

[a] Major bleeding defined as intracranial hemorrhage (ICH) or that leading to hemodynamic compromise in the PARAGON A and B trials, as a fall in hemoglobin (Hgb) × 5 g/dl, ICH,or cardiac tamponade in the PRISM study, as a fall in Hgb × 4 g/dl, need for 2 unit blood transfusion, need for corrective surgery, ICH or retroperitoneal bleed in PRISM-PLUS and according to TIMI criteria in PURSUIT.

[b] At 30 days, respective incidence of death in heparin and tirofiban groups: 3.6% vs. 2.3%, *P* = 0.02.

[c] Comparisons for tirofiban vs. heparin groups were made prior to discontinuation of tirofiban arm (heparin group data not shown).

[d] At 6 months, respective incidence of composite endpoint in heparin and tirofiban/heparin groups: 32.1 vs. 27.7%, *P* = 0.02.

[e] Any bleeding in tirofiban-heparin vs. placebo-heparin groups: 3.5% vs. 1.3%, *P* = 0.004.

[f] *p* values for low + hep, low – hep, high + hep and high – hep compared with hep alone at 30 days were 0.48, 0.66, 0.77 and 0.95, respectively; at 6 months, the low-dose groups had significant reductions in the composite endpoint (0.73 [95% CI 0.55–0.97]).

[g] At 6 months, low-dose (with or without heparin) lamifiban vs. placebo: 13.7% vs. 17.9% (OR 0.73 [95% CI 0.55–0.97]).

[h] Composite major or intermediate bleeding in high- and low-dose groups vs. placebo (10.7%, 6% and 5.5%, respectively, *P* = 0.002).

[i] At 6 months, respective incidence of death or non-fatal MI in placebo and eptifibatide groups: 19.0% vs. 17.8%.

[j] Respective incidence of death or non-fatal MI among placebo and eptifibatide groups in US cohort at 30 days: 15.4% vs. 11.9%, *P* = 0.003; 6 months: 18.9% vs. 15.2%, p = 0.004.

[k] At 30 days, among troponin positive patients, primary composite endpoint in placebo vs. lamifiban arms: 19.4% vs. 11.0%, *P* = 0.01; death or non-fatal MI: 19.0% vs. 11.0%, *P* = 0.02.

[l] Intermediate bleeding,11.5% vs. 14.0%, *P* = 0.002.

Cambridge Books Online © Cambridge University Press, 2009

for one month, streptokinase 150 million units intravenously over one hour, both or neither. Compared with placebo, aspirin significantly reduced the incidence of in-hospital non-fatal reinfarction (1.0% vs. 2.0%, $P<0.0000001$) and the incidence of the study's primary endpoint, vascular death at five weeks (9.4% vs. 11.8%; OR = 0.77 [95% CI 0.70–0.85], $P<0.00001$). Streptokinase also significantly reduced the incidence of the primary endpoint, but the combination of aspirin and streptokinase was not only superior to that of its placebo (8.0% vs. 13.2%; OR = 0.58 [95% CI 0.50–0.66], $P<0.00001$), but yielded significantly greater benefits than either agent alone. The mortality benefit in both aspirin groups persisted at 10 years of follow-up[79].

ADP receptor antagonists

To date, there have been no studies evaluating ADP receptor antagonists as monotherapy in the setting of ST elevation MI. However, when stenting is used as reperfusion therapy in this setting (i.e. primary stenting), these agents are routinely co-administered with aspirin (see below). In patients who receive either fibrinolysis or no reperfusion therapy and who do not undergo percutaneous revascularization, the relative benefits and risks of ADP receptor antagonism have not been explored. Given the benefits attributable to these agents in lower risk patients with CAD[33], such studies would be worthwhile.

GP IIb/IIIa inhibitors

Primary PCI with or without adjunctive abciximab

After encouraging preliminary observational data from the EPIC trial[66], the ReoPro and Primary PTCA Organization and Randomized Trial (RAPPORT) prospectively evaluated abciximab in the setting of primary PTCA (Table 69.5)[80]. At 6 months, the study's primary endpoint, death, MI or any target vessel revascularization (TVR) was not significantly different between abciximab and placebo groups. However, there was less death, MI or urgent TVR in the abciximab group. As expected, the incidence of major bleeding was greater among abciximab-treated patients.

In a small trial ($n = 200$), Neumann et al. demonstrated that the use of abciximab in primary stenting improved coronary microvascular function and left ventricular wall motion. Furthermore, it significantly reduced the incidence of the secondary endpoint, death, non-fatal MI or TVR at 30 days[81]. In the Abciximab before Direct Angioplasty and Stenting in Myocardial Infarction Regarding Acute and Long-term Follow-up (ADMIRAL) study, abciximab reduced the incidence of the study's primary endpoint, death, MI or urgent TVR at 30 days, a benefit that was preserved at 6 months (Table 69.5)[82]. Minor bleeding was more frequent among abciximab-treated patients as well.

In contrast, the Controlled Abciximab and Device Investigation to Lower Late Angioplasty Complications (CADILLAC) trial randomized patients to PTCA alone, PTCA + abciximab, stent alone or stent + abciximab and found that the study's primary endpoint, death, MI, disabling stroke or urgent TVR at six months, was significantly reduced by stenting but not by abciximab (Table 69.5)[83]. Bleeding rates were not different between any of the four treatment strategies. While the addition of abciximab appeared to decrease the incidence of major adverse cardiac events among patients treated with primary PTCA, it conferred no additional benefit in the setting of primary stenting. Of concern, the study endpoints were not consistent in direction among patients in the stent arms; while abciximab was associated with increased death and MI in these patients (a finding inconsistent with the weight of previous trial evidence), it substantially reduced ischemic TVR. In the end, these opposing benefits nullified one another and the overall incidence of major adverse cardiac events appeared equal in both stent groups. It is also noteworthy that the incidence of reinfarction was much lower than expected in the placebo arms; as a result, the overall number of cardiac events upon which the analysis was performed and from which the study conclusions were drawn was relatively small.

Primary stenting with adjunctive abciximab vs. full-dose thrombolysis

The Stent versus Thrombolysis for Occluded coronary arteries in Patients with Acute Myocardial Infarction (STOP–AMI) study randomized 140 patients to either primary stenting with adjunctive abciximab or thrombolysis using accelerated alteplase[84]. Primary stenting with abciximab was associated with greater myocardial salvage and a reduced incidence of the secondary endpoint, death, MI or stroke at 6 months (8.5% vs. 23.2%; RR 0.34, 95% CI 0.13–0.88, $P = 0.02$).

Full-dose thrombolysis with or without adjunctive abciximab, eptifibatide or lamifiban

Three phase II studies of full-dose thrombolysis with or without adjunctive GP IIb/IIIa inhibition for ST elevation MI have been conducted. The Thrombolysis and Angioplasty in Myocardial Infarction (TAMI) 8 Pilot[85], Integrilin to Minimize Platelet Aggregation and Coronary Thrombosis (IMPACT)-AMI[86] and Platelet Aggregation

Cambridge Books Online © Cambridge University Press, 2009

Table 69.5. Trials of platelet glycoprotein IIb/IIIa blockade for primary stenting

	RAPPORT	ADMIRAL	CADILLAC
Period of enrolment	11/95–02/97	NA	NA
Agent	Abciximab	Abciximab	Abciximab
Study size (*n*)	483	300	2665
Population	ST elevation MI <12 hours	ST elevation MI <12 hours	ST elevation MI <12 hours
Treatment group/s	Abciximab 0.25 mg/kg IV bolus, then 10 μg/min infusion × 12 hours initiated before, during or following coronary angiography (*n* = 241)	Abciximab 0.25 mg/kg IV bolus followed by 0.125 μg/kg/min infusion × 12 hours prior to the intervention (*n* = 150)	(1) PTCA + abciximab 0.25 mg/kg IV bolus pre-intervention, then 0.125 μg/kg/min infusion × 12 hours (*n* = 529) (2) Stent + placebo (*n* = 512) (3) Stent + abciximab 0.25 mg/kg IV bolus pre-intervention, then 0.125 μg/kg/min infusion × 12 hours (*n* = 525)
Control group	Placebo (*n* = 242)	Placebo (*n* = 150)	PTCA + placebo (*n* = 516)
Heparin dosing	Weight-adjusted heparin (100 U/kg) IV bolus (targeted to ACT300 s) continued for up to 48 hours (target aPTT 60–85 s)	NA	Heparin IV bolus/es (target ACT 200–300 s for PTCA and > 350 s for stent) discontinued immediately following procedure
Endpoint analysis	6 months	30-day	6 months
Primary endpoint	Death, MI or *any* TVR	Death, MI, TLR	Death, non-fatal MI, disabling stroke or urgent TVR

	RAPPORT			ADMIRAL			CADILLAC				
Outcome analysis	Placebo	Abciximab	*P* value[c]	Placebo	Abciximab	*P* value	PTCA	PTCA + Abx	Stent	Stent + Abx	*P* value
Primary endpoint (%)	28.1	28.2	0.90[d]	20	10.7	<0.03[a]	19.3	15.2	10.8	10.9	0.0001
Death (%)	4.5	4.1	0.82	4.7	3.3	NA	4.3	2.3	2.8	3.8	0.24
Non-fatal MI (%)	7.4	6.6	0.70	4.7	2.0	NA	1.6	2.1	1.2	2.3	0.53
Death or non-fatal MI (%)	11.2	8.7	0.36	NA	NA	NA	NA	NA	NA	NA	NA
Major bleeding (%)	9.5	11.6	0.02	2.6	4.0	0.50[b]	NA	NA	NA	NA	NA

Notes:

MI = myocardial infarction; IV = intravenous; ACT = activated clotting time; s = seconds; aPTT = activated partial thromboplastin time; TVR = target vessel revascularization; NA = not available; TLR = target lesion revascularization; PTCA = percutaneous transluminal coronary angioplasty.

[a] 30-day death, non-fatal MI or urgent TVR in placebo vs. abciximab groups: 14.7% vs. 7.3%, $P < 0.05$.

[b] The 30-day incidence of minor bleeding in the placebo and abciximab groups: 1.3% vs. 6.7%, $P = 0.02$.

[c] *P* values reflect an across-group comparison using the log-rank test.

[d] At 6 months, respective incidence of death, MI or urgent TVR in placebo vs. abciximab: 17.8% vs. 11.6%, $P = 0.048$.

Cambridge Books Online © Cambridge University Press, 2009

Receptor Antagonist Dose Investigation and Reperfusion Gain in Myocardial Infarction (PARADIGM)[87] trials assessed abciximab, eptifibatide and lamifiban for this purpose, respectively. Two of these studies demonstrated that adjunctive GP IIb/IIIa inhibition improved coronary macrovascular flow (as measured by Thrombolysis In Myocardial Infarction (TIMI) flow grade[88])[85,86], two revealed enhanced coronary microvascular flow (as measured by time to ECG ST segment recovery/stabilization)[86,87], but only one found that adverse clinical event rates were lower with the addition of these agents[85]. Not surprisingly, bleeding rates were greater among those patients treated with GP IIb/IIIa inhibitors in the TAMI-8 and PARADIGM trials.

Reduced-dose thrombolysis with adjunctive abciximab vs. full-dose thrombolysis

In the Phase II study, Thrombolysis In Myocardial Infarction (TIMI)-14, greater rates of 90-minute IRA patency (the study's primary endpoint) and more complete resolution of ECG ST segment elevation at 90 minutes were observed with reduced-dose alteplase and abciximab than with full-dose alteplase alone[89]. The Phase II Strategies for Patency Enhancement in the Emergency Department (SPEED) trial assessed whether the combination of reduced-dose reteplase with abciximab could safely provide superior reperfusion than full-dose reteplase alone. Reteplase was employed for thrombolysis as earlier data had demonstrated higher rates of reperfusion with this agent than alteplase[90]. Reduced-dose reteplase with abciximab yielded significantly greater 60–90 minute TIMI 3 grade flow than full-dose reteplase alone. Interestingly, TIMI 3 flow was achieved less often if low-dose rather than standard-dose heparin (40 vs. 60 U/kg intravenous bolus) was administered with the reduced-dose reteplase and abciximab regimen. Based on these findings, the GUSTO V-trial, is randomizing 16600 patients to full-dose reteplase vs. reduced-dose reteplase + abciximab with standard-dose heparin.

In summary, the evidence to date suggests that GP IIb/IIIa inhibitors improve angiographic and clinical outcomes when combined with thrombolysis and likely with primary PCI. Furthermore, primary stenting with adjunctive abciximab may be superior to full dose thrombolysis. Whether primary PCI with adjunctive GP IIb/IIIa receptor blockade is superior to reduced-dose thrombolysis with adjunctive GP IIb/IIIa inhibition remains to be seen.

Percutaneous coronary intervention

Aspirin with or without ADP receptor antagonists

Percutaneous transluminal coronary angioplasty

Adjunctive aspirin has been administered with percutaneous transluminal coronary angioplasty (PTCA) since this procedure was first performed in 1977. However, the first trial to demonstrate aspirin's clinical benefit in this setting did not appear until 1988[91]. In this study, aspirin given in combination with dipyridamole was associated with a significant reduction in the incidence of acute closure as manifest by lower rates of periprocedural Q-wave MI when compared with placebo (1.6% vs. 6.9%, $P = 0.011$).

Coronary stenting

Aspirin has been an integral component of the adjunctive pharmacologic regimen employed for coronary stenting as well[92,93]. Early pharmacologic regimens employed aspirin in combination with aggressive anticoagulation but were associated with poorer clinical outcomes and more procedural complications. At the other extreme, aspirin monotherapy is inadequate at preventing adverse clinical outcomes after coronary stenting. Although one small randomized trial suggested that aspirin in combination with ticlopidine was equivalent to aspirin monotherapy in preventing death, MI, revascularization or stent thrombosis, this study was underpowered[94]. The much larger Stent Anticoagulation Restenosis Study subsequently established the superiority of the aspirin–ticlopidine combination over aspirin alone. In that study, aspirin with ticlopidine significantly reduced the primary endpoint, death, TVR, target vessel thrombosis or recurrent MI when compared with aspirin monotherapy[95]. In addition, each of the individual component endpoints barring death were significantly reduced with aspirin–ticlopidine as compared with aspirin alone.

ADP receptor antagonists

Percutaneous transluminal coronary angioplasty with adjunctive ticlopidine

Few data are available on the use of ADP receptor antagonists in the setting of stand-alone PTCA. One small study by Bertrand et al. randomized 266 patients to ticlopidine 250 mg twice daily or placebo beginning 2 days prior to and continuing for 6 months following PTCA in order to assess the effect of ticlopidine on acute closure and angiographic restenosis[96]. While no significant differences were noted in the rate of restenosis between groups, those in the ticlopi-

Cambridge Books Online © Cambridge University Press, 2009

dine group had significantly fewer episodes of acute closure (5.1% vs. 16.2%, $P<0.01$).

Coronary stenting with adjunctive ticlopidine

The ADP receptor antagonists have played a much more integral role in the adjunctive pharmacotherapy for coronary stenting than stand-alone PTCA. Four randomized controlled trials comparing anticoagulation to antiplatelet therapy with ADP receptor antagonists have been published (Table 69.6). The Intracoronary Stenting and Antithrombotic Regimen (ISAR) trial[97] and Stent Anticoagulation Restenosis Study (STARS)[95] both demonstrated significant reductions in composite major adverse cardiac events, while in the Full ANTicoagulation vs. ASpirin and TIClopidine (FANTASTIC) trial[98] and Multicenter Aspirin and Ticlopidine Trial after Intracoronary Stenting (MATTIS)[99] non-significant reductions in these events were observed with antiplatelet therapy. Treatment with an ADP receptor antagonist reduced the incidence of MI, target lesion revascularization (TLR) and stent thrombosis by varying degrees in each of these trials, and significantly reduced the incidence of bleeding complications in all four. Finally, hospital length of stay (LOS) was significantly shortened by ADP receptor antagonism in FANTASTIC and MATTIS, the two trials in which this endpoint was measured.

Pre- vs. postprocedure ADP receptor antagonism

The duration of pretreatment with ADP receptor antagonists before coronary stenting also appears to influence the degree of protection from ischemic events conferred by these agents. In a Cleveland Clinic registry study of 175 patients who underwent coronary stenting, Steinhubl et al. observed that the rate of procedure-related non-Q MI varied with the duration of ticlopidine pretreatment[100]. Among those who began taking ticlopidine on the day of procedure, 1 to 2 days preprocedure and greater than 3 days before the procedure, the respective rates of post-procedure non-Q MI were 29%, 14% and 5% (chi-square for trend $=9.6$, $P=0.002$). A TARGET trial (see below) substudy, examining the effect of clopidogrel pretreatment is under way.

Coronary stenting with adjunctive ticlopidine vs. clopidogrel

Despite the significant reductions in thrombotic and hemorrhagic complications following coronary stenting afforded by combination antiplatelet therapy compared to oral anticoagulation, ticlopidine use is limited by significant hematological and gastrointestinal side effects[18]. As a result, clopidogrel, a newer ADP receptor antagonist with a more rapid onset of action, greater inhibition of platelet aggregation and more tolerable side effect profile[18] has also been tested in this setting. Ten studies have compared the combination of ASA and ticlopidine with ASA and clopidogrel in patients undergoing coronary stenting. Considered individually, data from these three randomized trials[101–103] and seven registries[104–110] have demonstrated that the combination of clopidogrel and ASA is safer and at least as efficacious in preventing major adverse cardiac events following coronary stenting than combined ticlopidine and ASA. When these studies were meta-analysed, clopidogrel was associated with significant reductions in the incidence of major adverse cardiac events (OR 0.50, 95% CI 0.41–0.62, $P=0.001$), including mortality (OR 0.43, 95% CI 0.28–0.66, $P=0.001$) as compared with ticlopidine[111]. Consequently, most interventional cardiologists have now switched from ticlopidine to clopidogrel for patients undergoing coronary stenting.

GP IIb/IIIa receptor blockade for percutaneous coronary intervention

Platelets play a central role in the development of ischemic complications following percutaneous interventions[112,113], and therapeutic intravenous GP IIb/IIIa receptor blockade was first explored in this setting (Table 69.7).

Abciximab

The first clinical trial demonstrating the benefit of GP IIb/IIIa receptor blockade in any patient population was the Evaluation of c7E3 for the Prevention of Ischemic Complications (EPIC). EPIC randomly assigned patients undergoing PTCA or DCA who were at high-risk for abrupt closure to an abciximab (C7E3) bolus and infusion, abciximab bolus with placebo infusion, or placebo bolus and infusion[114]. Compared with the placebo bolus and infusion, the abciximab bolus and infusion was associated with a significant reduction in major ischemic events by 30 days, a difference largely due to reductions in non-fatal MI and emergent PTCA. Nevertheless, major bleeding was twice as common with abciximab than placebo. The clinical benefits were durable at 6 months[115] and 3 years[116]. Notably, among those enrolled with unstable angina or evolving MI, the highest risk patient subgroup in that trial, abciximab treatment was associated with a substantial reduction in 3-year mortality (5.1% vs. 12.7%, $P=0.01$).

The Evaluation of PTCA to Improve Long-Term Outcome with Abciximab GP IIb/IIIa Blockade (EPILOG) trial was conducted to discern whether GP IIb/IIIa receptor blockade would benefit a broad range of patients undergoing PCI, and whether using less heparin could reduce

Cambridge Books Online © Cambridge University Press, 2009

Table 69.6. Randomized trials of anticoagulation vs. ticlopidine for coronary stenting

	ISAR	FANTASTIC	STARS	MATTIS
Period of enrolment	10/94–09/95	05/95–05/96	02/96–11/96	02/96–01/97
Study size (*n*)	517	485	1653	350
Population	Successful stenting for symptomatic stable or unstable coronary syndromes in intermediate risk patients	Attempted planned or unplanned stenting in intermediate risk patients	Successfully stented low risk patients (excluded acute MI)	High risk patients
Treatment group/s	Ticlopidine 250mg twice daily × 4 weeks beginning postprocedure (*n* = 257)	Ticlopidine 500 mg oral load in cath lab, then 250 mg twice daily × 6 weeks (*n* = 243)	Ticlopidine 250 mg postprocedure and then twice daily thereafter × 4 weeks (*n* = 546)	Ticlopidine 500 mg oral load within 6 hours postprocedure, then 250 mg twice daily (*n* = 177).
Control group	Phenprocoumaron (target INR 3.5–4.5) × 4 weeks (*n* = 260)	Oral anticoagulation (target INR 2.5–3.0) × 6 weeks (*n* = 230)	(1) Aspirin only (*n* = 557) (2) Warfarin (target INR 2.0–2.5) × 4 weeks (*n* = 550)	Oral anticoagulation (target INR 2.5–3.0) (*n* = 173)
ASA dosing	Aspirin 500 mg preprocedure; 100 mg twice daily post-procedure	Aspirin 100–325 mg preprocedure and daily thereafter	Aspirin 325 mg preprocedure and daily thereafter	Aspirin 250 mg within 6 hours postprocedure, then daily
Heparin dosing	Heparin 15 000 U IV bolus pre-procedure. Infusion (target aPTT 80–100 s) discontinued 12 hours postprocedure in ticlopidine group; continued 5–10 days postprocedure in phenprocoumaron group	Heparin 10 000 U IV bolus preprocedure with supplemental boluses as needed; no postprocedure heparin in ticlopidine group if same-day sheath removal; infusion continued in anticoagulation arm (target aPTT 2.0–2.5 × normal) until therapeutic INR × 2 days	Heparin 10–15 000 U IV bolus pre-procedure (target ACT 250–300 s); in warfarin arm, infusion continued (target aPTT 40–60 s) until therapeutic INR	Heparin infusion discontinued 6 hours before sheath removal in ticlopidine group and continued (target aPTT 2.0–2.5 × normal) until therapeutic INR × 2 days in anticoagulation arm
Stent	Palmaz–Schatz	Wiktor	Palmaz–Schatz	Per investigators' discretion
Endpoint analysis	30 days	6 weeks	30 days	30 days
Primary endpoint	Cardiac death, MI or TVR	Any bleeding	Death, TLR, target vessel thrombosis or non-fatal MI	Cardiovascular death, non-fatal MI or TLR

	ISAR			FANTASTIC			STARS					MATTIS		
Outcome analysis	OAC	T	*P* value	OAC	T	*P* value	ASA	T	*P* value[d]	OAC	*P* value[e]	OAC	T	*P* value
Primary endpoint (%)	6.2	1.6	0.01	21.0	13.5	0.03	3.6	0.5	<0.001	2.7	0.01	11.0	5.6	0.07
Death (%)	0.8	0.4	1.0	1.7	0.8	0.37	0.2	0	–	0	–	1.2	1.7	0.67[c]
Non-fatal MI (%)	3.5	0.8	0.06	6.5	4.9	0.46[c]	2.7	0.5	0.014	2.0	0.11	6.9	3.4	0.13[c]
TLR%	5.4	1.2	0.01[b]	NA	NA	NA	3.4	0.5	0.001	2.5	0.02	8.1	3.4	0.06[c]
Stent thrombosis (%)	5.0	0	<0.001	3.9	2.8	0.53	2.9	0.5	0.001	2.7	0.01	NA	NA	NA
Major bleeding (%)[a]	6.5	0	<0.001	21.0	13.5	0.03	1.8	5.5	0.002	6.2	0.99	6.9	1.7	0.02
Length of stay (days)	NA	NA	NA	6.4	4.3	0.0001	NA	NA	NA	NA	NA	7.7	4.6	<0.0001

Notes:

MI = myocardial infarction; TLR = target lesion revascularization; INR = international normalized ratio; IV = intravenous; aPTT = activated partial thromboplastin time; OAC = oral anticoagulation; T = ticlopidine; NA = not available; ASA = aspirin.

[a] Bleeding defined as requiring surgical correction, transfusion or causing organ dysfunction in ISAR, as requiring blood transfusion in STARS, bleeding of any kind in FANTASTIC, and as one or more of the following in MATTIS: surgical repair of the vascular access site, decrease in hemoglobin ≥4 g/dL and/or requiring ≥2U blood transfusion, intracranial or retroperitoneal hemorrhage.

[b] These data reflect TVR.

[c] *P* values not provided; calculated using Cochran Mantel–Haenszel chi square test.

[d] Aspirin + ticlopidine compared with ASA alone.

[e] Aspirin + ticlopidine compared with ASA + warfarin.

Cambridge Books Online © Cambridge University Press, 2009

Table 69.7. Trials of platelet glycoprotein IIb/IIIa inhibitors for percutaneous coronary intervention

	EPIC	CAPTURE	IMPACT-II	RESTORE
Period of enrolment	11/91–11/92	05/93–12/95	11/93–11/94	01/95–12/95
Agent	Abciximab	Abciximab	Eptifibatide	Tirofiban
Study size (n)	2099	1265	4010	2141
Population	High-risk for abrupt closure	Medically refractory unstable angina	Elective, urgent or emergent intervention	Within 72 hours of ACS
Treatment group/s	(1) Abciximab 0.25 mg/kg IV bolus (n = 695) (2) Abciximab 0.25 mg/kg IV bolus 10 minutes preprocedure, then 10 μg/min infusion × 12 hours (n = 708)	Abciximab 0.25 mg/kg IV bolus, then 10 μg/min infusion × 18–24 hours preprocedure and × 1 h postprocedure (n = 630).	(1) Eptifibatide 135 μg/kg IV bolus 10–60 minutes preprocedure, then 0.5 μg/kg/min infusion × 20–24 hours (n = 1349) (2) Eptifibatide 135 μg/kg IV bolus, 0.75 μg/kg/min infusion for 20–24 hours (n = 1333)	Tirofiban 10 μg/kg IV bolus over 3 minutes, then 0.15 μg/kg/min infusion × 36 hours (n = 1071)
Control group	Placebo (n = 696)	Placebo (n = 635)	Placebo (n = 1328)	Placebo (n = 1070)
Aspirin/ADP receptor antagonist dosing	Aspirin 325 mg ≥2 hours preprocedure and daily thereafter	Aspirin 50–250 mg oral load preprocedure and ≥50 mg daily thereafter	Aspirin 325 mg preprocedure, indefinitely thereafter	Aspirin 325 mg ≤12 hours preprocedure
Heparin dosing	Heparin 10–12 000 U IV bolus (target ACT 300–350 s) and infusion (target aPTT 1.5–2.5 × normal) × ≥12 hours	Heparin 100U/kg IV bolus (target ACT >300 s) adjusted to keep aPTT 2–2.5 × normal continued ≥1 hour postprocedure	Heparin 100U/kg IV bolus (target ACT 300–350 s) discontinued immediately following procedure	Heparin 150U/kg IV bolus (target ACT 300–400 s) preprocedure. Discontinued immediately postprocedure
PCI	PTCA or DCA	PTCA	PTCA, DCA, RCA, excimer laser angioplasty	PTCA, DCA
Endpoint analysis	30 days	30 days	30 days	30 days
Primary endpoint	Death, nonfatal MI, refractory ischemia, emergent revascularization, stent placement, IABP insertion	Death, non-fatal MI or urgent revascularization	Death, MI, urgent/emergent revascularization, or stent for abrupt or threatened closure	Death, non-fatal MI, stent for abrupt/threatened closure, any revascularization

Outcome analysis	Placebo	Abx bolus	Abx bolus/gtt	P value[b]	Placebo	Abx	P value	Placebo	Ept 135/5	P value	Ept 135/75	P value	Placebo	Tirofiban	P value
Primary endpoint (%)	12.8	11.4	8.3	0.009[c]	15.9	11.3	0.012	11.4	9.2	0.063[f]	9.9	0.22[f]	12.2	10.3	0.16[g]
Death (%)	1.7	1.3	1.7	0.96	1.3	1.0	>0.1	1.1	0.5	P = NS	0.8	P = NS	0.7	0.8	0.81
Non-fatal MI (%)	8.6	6.2	5.2	0.013	8.2	4.1	0.002	8.1	6.6	P = NS	6.9	P = NS	5.7	4.2	0.11
Death or non-fatal MI (%)	NA	NA	NA	NA	9.0	4.8	0.003	8.4	6.9	0.13	7.3	0.27	NA	NA	NA
Urgent revascularization (%)	4.5	3.6	0.8	<0.001[d]	10.9	7.8	0.054	2.8	2.6	P = NS[d]	2.9	P = NS[d]	5.4	4.2	0.18[d]
Major bleeding (%)[a]	6.6	10.9	14.0	0.001[e]	19.0	3.8	0.043	4.8	5.1	P = NS	5.2	P = NS	3.7	5.3	0.096

Cambridge Books Online © Cambridge University Press, 2009

	EPILOG	EPISTENT	ESPRIT	TARGET
Period of enrolment	02/95–12/95	07/96–09/97	NA	12/99–08/00
Agent	Abciximab	Abciximab	Eptifibatide	Tirofiban
Study size (n)	2792	2399	2064	4812
Population	Elective or urgent intervention, excluding ACS or planned stent/DCA	Elective or urgent PCI, excluding acute MI	Elective coronary stenting	Stable CAD or unstable ACS
Treatment group/s	(1) Abciximab 0.25 mg/kg IV bolus 10–60 minutes preprocedure, then 0.125 μg/kg/min infusion × 12 hours; standard-dose heparin (n = 918) (2) 0.25 mg/kg IV bolus 10–60 minutes preprocedure, then 0.125 μg/kg/min infusion × 12 hours; low-dose heparin (n = 935)	(1) Stent + abciximab 0.25 mg/kg IV bolus up to 60 minutes preprocedure, then 0.125 μg/kg/min infusion × 12 hours; low-dose heparin (n = 794) (2) PTCA + abciximab 0.25 mg/kg IV bolus up to 60 minutes preprocedure, then 0.125 μg/kg/min infusion × 12 hours; low-dose heparin (n = 796)	Eptifibatide 180 μg/kg bolus, second eptifibatide 180 μg/kg bolus 10 min after the first, then 2.0 μg/kg /min infusion × 18–24 hours (n = 1040)	Abciximab 0.25 mg/kg IV bolus, then 0.125 μg/kg/min × 12 hours (n = 2414)
Control group	Placebo (n = 939)	Stent + Placebo; standard-dose heparin (n = 809)	Placebo (n = 1024)	Tirofiban 10 μg/kg IV bolus, then 0.15 μg/kg/min × 18–24 hours (n = 2398)
Aspiririn/ADP receptor antagonist dosing	Aspirin 325 mg 2 hours preprocedure and daily thereafter	Aspirin 325 mg 2 hours preprocedure and daily thereafter; ticlopidine 250 mg twice daily in stented patients	All received aspirin	All received aspirin; clopidogrel 300 mg oral load pre-PCI, then 75 mg daily × 29 days
Heparin dosing	(1) Standard-dose: Heparin 100 U/kg IV bolus (target ACT > 300 s) discontinued immediately postprocedure (2) Low-dose 70 U/kg heparin IV bolus (target ACT > 200 s) discontinued immediately postprocedure	(1) Standard-dose: Heparin 100 U/kg IV bolus (target ACT > 300 s) discontinued immediately postprocedure (2) Low-dose: Heparin 70U/kg IV bolus (target ACT > 200 s) discontinued immediately postprocedure	Heparin 60 U/kg IV bolus (target ACT 200–300 s)	Heparin 70 U/kg IV bolus (target ACT 250 s) discontinued immediately postprocedure
PCI	PTCA, DCA, or excimer laser angioplasty	As randomized per protocol	Stent	Stent
Endpoint analysis	30 days	30 days	48 hours	30 days
Primary endpoint	Death, non-fatal MI or urgent revascularization	Death, non-fatal MI or urgent revascularization	Death, MI, urgent TVR or need for bail-out GP IIb/IIIa receptor blockade	Death, non-fatal MI or urgent TVR

Cambridge Books Online © Cambridge University Press, 2009

Table 69.7. (*cont.*)

	EPIC	CAPTURE		IMPACT-II		RESTORE										
Outcome analysis	Placebo	Abx-Low	*P* value	Abx-High	*P* value	Stent-Pbo	PTCA-Abx	*P* value	Stent-Abx	*P* value	Placebo	Eptifibatide	*P* value	Tirofiban	Abciximab	*P* value
Primary endpoint (%)	11.7	5.2	<0.001	5.4	<0.001	10.8	6.9	0.007	5.3	<0.001	10.5	6.6	0.0015	7.55	6.01	0.037
Death (%)	0.8	0.3	0.21	0.4	0.39	0.6	0.8	*P* = NS	0.3	*P* = NS	0.2	0.1	0.55	0.50	0.41	*P* = NS
Non-fatal MI (%)	8.7	3.7	<0.001	3.8	<0.001	9.6	5.3	<0.05	4.5	<0.05	9.0	5.4	0.0015	6.88	5.43	0.04
Death or non-fatal MI (%)	9.1	3.8	<0.001	4.2	<0.001	7.8	4.7	0.010	3.0	<0.001	9.2	5.5	0.0013	7.17	5.72	0.043
Urgent revascularization (%)	5.2	1.6	<0.001	2.3	<0.001	2.1	1.9	*P* = NS	1.3	*P* = NS	1.0	0.6	0.30	0.83	0.66	*P* = NS
Major bleeding (%)[a]	3.1	2.0	0.19	3.5	0.70[h]	2.2	1.4	NA	1.5	NA	0.4	1.3	0.02[i]	1.17	0.95	0.47

Notes:

ADP = adenosine diphosphate; PCI = percutaneous coronary intervention; MI = myocardial infarction; IV = intravenous; ACT = activated clotting time; sec = seconds; aPTT = activated partial thromboplastin time; PTCA = percutaneous transluminal coronary angioplasty; DCA = directional coronary atherectomy; IABP = intra-aortic balloon pump; abx = abciximab; NA = not available; RCA = rotational coronary atherectomy; ept = eptifibatide; NS = non-significant; ACS = acute coronary syndrome; TVR = target vessel revascularization; CAD = coronary artery disease.

[a] Major bleeding defined according to the Thrombolysis in Myocardial Infarction criteria as ICH or decrease in hemoglobin ≥5 g/dl in EPIC, CAPTURE, IMPACT-II, EPILOG, EPISTENT, ESPRIT and TARGET; as decrease in hemoglobin ≥5 g/dl, transfusion 2 U of blood, corrective surgery, ICH or retroperitoneal bleed in RESTORE;

[b] *P* value for trend.

[c] For comparison of abciximab bolus and infusion vs. placebo groups, $P = 0.008$; for comparison of bolus only vs. placebo, $P = 0.43$.

[d] Comparison reflects PCI only (i.e. not CABG).

[e] For comparison of abciximab bolus and infusion vs. placebo groups only.

[f] On-treatment analysis of 30-day primary endpoint: placebo vs. 135/0.5 (11.6% vs. 9.1%, $P = 0.035$); placebo vs. 135/0.75 (11.6% vs. 10.0%, $P = 0.18$).

[g] When only urgent/emergent revascularization included in 30-day composite primary endpoint, placebo vs. tirofiban (10.5% vs. 8.0%, $P = 0.052$)

[h] Minor bleeding in placebo, low-dose and high-dose groups: 3.7% vs. 4.0% vs. 7.4% ($P < 0.001$and $P = 0.81$ for high-dose and low-dose vs. placebo, respectively).

[i] *P* value not available; calculated using Mantel–Haenszel chi-square test.

Cambridge Books Online © Cambridge University Press, 2009

hemorrhagic complications without attenuating the clinical benefit. EPILOG randomly assigned patients to a bolus and infusion of placebo with standard-dose heparin, abciximab with standard-dose heparin or abciximab with low-dose heparin. By 30 days, abciximab treatment had significantly reduced the incidence of ischemic events by more than half, regardless of the heparin dosing strategy. Although major bleeding did not vary by treatment assignment, significantly more minor bleeding was observed in the abciximab + standard-dose heparin than control group (7.4% vs. 3.7%, $P < 0.001$). Between 30 days and six months, some attenuation in benefit occurred due to abciximab's lack of effect on non-urgent TVR (a finding in direct contrast with the EPIC trial). Nevertheless, the incidence of the composite, death, MI or repeat revascularization at 6 months was reduced from 25.8% in the placebo group, to 22.3% in the abciximab + standard-dose heparin group ($P = 0.04$) and 22.8% in the abciximab + low-dose heparin group ($P = 0.07$).

While the EPIC and EPILOG studies examined the effect of abciximab during and following a PCI, the C7E3 Fab AntiPlatelet Therapy in Unstable REfractory angina (CAPTURE) trial[67] assessed the effect of prolonged preprocedural abciximab in medically refractory unstable angina. The trial was terminated early due to the significant reduction in the 30-day ischemic endpoint afforded by abciximab. However, by 6 months, there was no longer a significant difference in the incidence of ischemic events between abciximab and placebo arms. Abciximab pretreatment reduced the incidence of MI before the index PCI, a finding that suggested abciximab might be of benefit for unstable coronary syndromes regardless of the ultimate treatment strategy. As noted above, this hypothesis was not borne out in the subsequent GUSTO IV-ACS trial.

Very few patients in the EPIC, EPILOG and CAPTURE trials underwent coronary stenting. Given the superiority of stenting over PTCA for preventing repeat revascularization[92,93], the Evaluation of Platelet IIb/IIIa Inhibitor for STENTing (EPISTENT) trial randomized patients to stent + abciximab, PTCA + abciximab or stent + placebo[65]. The incidence of death, non-fatal MI or urgent revascularization was significantly reduced in the stent + abciximab group and in the PTCA + abciximab group when compared with the stent + placebo group. These results were durable at six months[117] and 1 year, and were largely the result of reductions in death and MI afforded by abciximab[118]. Perhaps, most notably, the death rate at 1 year was significantly lower in the stent + abciximab group than the stent + placebo group (2.4% vs. 1.0%, $P = 0.037$), additional data suggesting that GP IIb/IIIa blockade might confer a survival advantage in the setting of PCI.

Despite the suggestion that GP IIb/IIIa inhibition with abciximab might reduce the incidence of clinical restenosis in the EPIC trial[115], these findings were not borne out in the subsequent EPILOG[119] or EPISTENT[117] studies, excepting diabetics, who witnessed a significant reduction in TVR at 6 months in the latter trial (22.4% vs. 13.7%, $P = 0.035$)[120]. The Evaluation of ReoPro And Stenting to Eliminate Restenosis (ERASER) trial randomized approximately 200 patients to a bolus and infusion of abciximab or placebo at the time of coronary stenting and found no difference in the primary endpoint, restenotic tissue volume by intravascular ultrasound (there was a non-significant trend toward reduced volume of restenotic tissue among abciximab-treated diabetics, however)[121]. Similarly, the Intracoronary Stenting and Antithrombotic Regimen-2 (ISAR-2) trial randomized patients undergoing coronary stenting within 48 hours of an acute ST elevation MI to abciximab or placebo bolus and infusion and found no difference in the primary endpoint, angiographic restenosis at 6 months among 366 patients[122]. Thus, to date, the only data supporting the role of GP IIb/IIIa receptor blockade for inhibiting restenosis are in diabetics.

Eptifibatide

After promising pilot data on eptifibatide in the setting of PCI[123,124], patients undergoing elective, urgent or emergent intervention in the Integrilin to Minimize Platelet Aggregation and Coronary Thrombosis (IMPACT)-II study were randomized to an eptifibatide bolus with moderate-dose infusion, eptifibatide bolus with low-dose infusion or a placebo bolus and infusion[125]. At 30 days, there was a gradient of benefit, albeit non-significant, with increasing eptifibatide dose and no statistically significant difference in the rates of bleeding complications. In an on-treatment analysis, the composite endpoint, death, MI, urgent revascularization or placement of a stent for abrupt or threatened closure was significantly reduced in the low-dose eptifibatide group when compared with placebo.

The Enhanced Suppression of the Platelet Receptor glycoprotein IIb/IIIa using Integrilin Therapy (ESPRIT) trial sought to determine whether eptifibatide would confer benefit in patients undergoing elective coronary stenting. Utilizing a novel dosing strategy aimed at attaining greater platelet inhibition, this trial randomized patients to a double bolus and infusion of eptifibatide or placebo. At 48 hours, the study's primary endpoint, death, MI, urgent TVR or bailout GP IIb/IIIa inhibitor use, was significantly reduced. By 30 days, the composite secondary endpoint, death, MI or urgent TVR was significantly reduced by eptifibatide (6.8% vs. 10.5%, $P = 0.003$), a difference largely driven by reductions in death and MI. Finally, a posthoc analysis of the angiographic data from 43 patients in this

Cambridge Books Online © Cambridge University Press, 2009

trial revealed that eptifibatide also significantly improved coronary flow reserve when compared with placebo (1.78 ± 0.95 vs. 1.28 ± 0.40, $P = 0.02$)[126]. Six-month trial results are not yet available. While it appears that eptifibatide reduces major adverse cardiac events following PCI, its efficacy relative to other GP IIb/IIIa inhibitors in this setting has not yet been studied.

Tirofiban

The Randomized Efficacy Study of Tirofiban for Outcomes and Restenosis (RESTORE) was designed to assess the effect of tirofiban in PCI. RESTORE enrolled patients undergoing PTCA or DCA within 72 hours of USA or an MI and randomized them to tirofiban or placebo bolus and infusion[127]. The incidence of death, MI, stent for threatened/abrupt closure or any revascularization at 30 days was non-significantly reduced in the tirofiban-treated group and bleeding complications were similar in the two treatment groups. When the composite endpoint, death, non-fatal MI or urgent/emergent revascularization was examined to allow for comparison with trials of other GP IIb/IIIa inhibitors in this setting, the event rate in the tirofiban group was reduced by 24%, although this result was of only marginal statistical significance. At 6 months, the incidence of the primary endpoint remained lower in the tirofiban group (24.1% vs. 27.1%, $P = 0.11$)[128].

Head-to-head comparison of GP IIb/IIIa inhibition in PCI

The role of tirofiban in PCI was further examined in the equivalency trial, do Tirofiban and Abciximab for Revascularization Give Equivalent outcomes? (TARGET)[129]. This study randomized patients undergoing coronary stenting to either abciximab or tirofiban and found that abciximab was superior to tirofiban in preventing the composite endpoint, death, MI or urgent revascularization at 30 days. No trials of abciximab or tirofiban vs. eptifibatide for PCI are currently under way.

Note added in proof

A number of noteworthy developments in the antiplatelet therapy arena occurred following submission of this chapter. Important trials in primary and secondary prevention, non-ST and ST elevation ACS and PCI have subsequently been published or presented and are reviewed below:

Primary prevention

The Primary Prevention Project (**PPP**) randomized 4495 people who had at least one cardiovascular disease risk factor but no known coronary heart disease to either aspirin 100 mg daily or placebo.[130] It was stopped prematurely after a mean of 3.6 years after the TPT[22] and HOT[23] trials revealed that aspirin therapy was of unequivocal benefit in the primary prevention of cardiovascular disease. In the PPP, aspirin therapy significantly lowered the risk of cardiovascular death compared with placebo (0.8% vs. 1.4%, RR 0.56 [95% CI 0.31–0.99], $P = 0.049$). In addition, the composite cardiovascular death, non-fatal MI, angina, non-fatal stroke, transient ischemic attack, peripheral arterial disease or revascularization procedures was also significantly lowered among patients assigned to aspirin treatment (6.3% vs. 8.2%, RR 0.77 [95% CI 0.62–0.95], $P = 0.014$). Importantly, while bleeding complications were more frequent among aspirin-treated patients (1.1 vs. 0.3%, $P = 0.0008$), aspirin's observed benefit did not occur at the expense more hemorrhagic cerebrovascular events.

Secondary prevention

After enrolling 9200 patients, the Blockade of the IIb/IIIa Receptor to Avoid Vascular Occlusion (**BRAVO**) trial was prematurely terminated per the recommendation of the independent Data and Safety Monitoring Board. Compared with placebo, lotrafiban was associated with a significant increase in mortality (2.7% vs. 2.0%, $P = 0.022$), serious thrombocytopenia (2.2% vs. 0.5%) and major bleeding (4.2% vs. 1.3%, $P < 0.0001$). Given similar findings in studies of sibrafiban, orbofiban and zemilofiban, trials of chromofiban and roxifiban been terminated and there are currently no ongoing phase III trials of any oral glycoprotein IIb/IIIa inhibitor.

In the Clopidogrel in Unstable Angina to Prevent Recurrent Events (**CURE**) trial, clopidogrel treatment was added to background aspirin therapy for 3–12 months following a non-ST elevation ACS.[131] At 12 months, clopidogrel significantly reduced the primary composite endpoint, cardiovascular death, non-fatal MI or stroke (9.3% vs. 11.4%, RR 0.80 [0.72–0.90], $P < 0.001$). The occurrence of any composite endpoint component or refractory ischemia was also significantly reduced (16.5% vs. 18.8%, RR 0.86 [0.79–0.94], $P < 0.001$). Nevertheless, minor (5.1% vs. 2.4%, RR 2.12 [1.75–2.56], $P < 0.001$) and major (3.7% vs. 2.7%, RR 1.38 [1.13–1.67], $P = 0.001$) bleeding, and the need to transfuse two or more units of blood (2.8% vs. 2.2%, RR 1.30 [1.04–1.62], $P = 0.02$) occurred more frequently among patients randomized to clopidogrel therapy.

Non-ST elevation ACS

The **TACTICS** Thrombolysis In Myocardial Infarction (**TIMI**)**-18** trial randomized 2220 patients with a non-ST elevation ACS to an early invasive or conservative

Cambridge Books Online © Cambridge University Press, 2009

approach.[132] All patients were treated with aspirin, heparin and tirofiban (the latter for 48 hours or until revascularization and then for 12 hours following PCI). The early invasive strategy was associated with a significantly lower incidence of the primary composite endpoint, death, non-fatal MI or rehospitalization for an ACS within six months when compared with the conservative strategy (15.9% vs. 19.4%, OR 0.97 [0.62–0.97], *P* = 0.025). While the trial did not specifically assess the efficacy of tirofiban, it was remarkable in that an early invasive strategy with background tirofiban was associated with a 4.7% incidence of death or non-fatal MI at 30 days, a rate lower than in prior trials of patients with non-ST elevation ACS.

ST elevation ACS

The Global Use of Strategies To Open occluded coronary arteries (**GUSTO**) **V** trial randomized 16,588 patients who presented within six hours of an ST elevation MI to either standard-dose reteplase or half-dose reteplase with full-dose abciximab.[133] Although the incidence of the trial's primary endpoint, all-cause mortality at 30-days, was lower among patients treated with combination therapy, this difference was not statistically significant (5.6% vs. 5.9%, RR 0.95 [95% CI 0.84–1.08], *P* = 0.43). Nevertheless, the rates of the composite endpoint death or non-fatal MI (7.4% vs. 8.8%, RR 0.83 [0.74–0.93], *P* = 0.0011) were significantly reduced by combination therapy. Although the incidence of intracranial bleeding was similar in each group, mild, moderate and severe bleeding complications were nearly twice as common, and the development of thrombocytopenia and need for blood transfusion were also significantly increased among patients treated with combination therapy.

The Assessment of the Safety and Efficacy of a New Thrombolytic (**ASSENT**)**-3** study randomized 6,095 patients with an ST elevation MI to one of three treatment strategies: full-dose tenecteplase plus *enoxaparin*; half-dose tenecteplase plus weight-adjusted low-dose unfractionated heparin and *abciximab*; or, full-dose tenecteplase plus weight-adjusted *unfractionated heparin*.[134] As in GUSTO V, 30-day mortality did not vary by treatment group. However, after adjusting for multiple statistical comparisons, the composite primary efficacy endpoint 30-day death, in-hospital non-fatal MI or in-hospital refractory ischemia was significantly reduced in both the enoxaparin and abciximab groups compared to the unfractionated heparin group (11.4% enoxaparin vs. 15.4% unfractionated heparin, RR 0.74 [95% CI 0.63–0.87], *P* = 0.0002; 11.1% abciximab vs. 15.4% unfractionated heparin, RR 0.72 [0.61–0.84], *P* = 0.0009). After similar adjustments, the composite efficacy and safety endpoint (primary efficacy endpoint plus intracranial bleeding or major in-hospital bleeding complications) was significantly reduced in the enoxaparin but only marginally reduced in the abciximab group compared with the unfractionated heparin group (13.7% enoxaparin vs. 17.0% unfractionated heparin, RR 0.81 [95% CI 0.70–0.93], *P* = 0.0146; 14.2% abciximab vs. 17.0% unfractionated heparin, RR 0.84 [95% CI 0.72–0.96], *P* = 0.057).

Thus, the addition of glycoprotein IIb/IIIa inhibitor therapy to thrombolysis not only improves patency in the infarct-related epicardial vessel and enhances tissue-level perfusion in the downstream myocardium, but appears to reduce combined morbidity and mortality as well. Nevertheless, these benefits occurred at the cost of more frequent thrombocytopenia, bleeding complications and need for blood transfusion.

PCI

In the **TARGET** trial, while abciximab was superior to tirofiban in preventing the composite endpoint death, non-fatal MI or urgent target vessel revascularization at 30 days,[135] these treatments appeared to be equally efficacious at six months (14.3% abciximab vs. 14.8% tirofiban, RR 1.04 [95%CI 0.90–1.21], *P* = 0.591).

In the **CURE** trial **PCI** substudy, 1313 patients were randomized to clopidogrel and 1345 to placebo.[136] In addition to aspirin, patients were pretreated with study drug for a median of six days prior to their PCI and the majority given an open-label thienopyridine for four weeks afterward. Study drug was subsequently reinitiated for an additional eight months on average. Clopidogrel treatment significantly reduced the incidence of the primary composite endpoint, death, non-fatal MI or urgent target vessel revascularization at 30 days compared with placebo (4.5% vs. 6.4%, RR 0.70 [95% CI 0.50–0.97], *P* = 0.03). The incidence of MI prior to the PCI was also significantly reduced by clopidogrel treatment (3.6% vs. 5.1%, RR 0.68 [0.47–0.99], *P* = 0.04). While minor bleeding complications were more frequent among clopidogrel-treated patients (3.5% vs. 2.1%, RR 1.68 [1.06–2.68], *P* = 0.03), the incidence of major bleeding and the need for blood transfusion were no different between groups.

References

1 Aronow WS, Ahn C, Kronzon I, Gutstein H. Effect of warfarin versus aspirin on the incidence of new thromboembolic stroke in older persons with chronic atrial fibrillation and abnormal and normal left ventricular ejection fraction. *Am J Cardiol* 2000; 85: 1033–1035.

2 Al-Khadra AS, Salem DN, Rand WM, Udelson JE, Smith JJ, Konstam MA. Antiplatelet agents and survival: a cohort

Cambridge Books Online © Cambridge University Press, 2009

analysis from the Studies of Left Ventricular Dysfunction (SOLVD) trial. *J Am Coll Cardiol* 1998; 31: 419–425.
3 Altman R, Rouvier J, Gurfinkel E, Scazziota A, Turpie AG. Comparison of high-dose with low-dose aspirin in patients with mechanical heart valve replacement treated with oral anticoagulant. *Circulation* 1996; 94: 2113–2116.
4 Meschengieser SS, Fondevila CG, Frontroth J, Santarelli MT, Lazzari MA. Low-intensity oral anticoagulation plus low-dose aspirin versus high- intensity oral anticoagulation alone: a randomized trial in patients with mechanical prosthetic heart valves. *J Thorac Cardiovasc Surg* 1997; 113: 910–916.
5 Blair KL, Hatton AC, White WD et al. Comparison of anticoagulation regimens after Carpentier-Edwards aortic or mitral valve replacement. *Circulation* 1994; 90: II214–219.
6 Turpie AG, Gent M, Laupacis A et al. A comparison of aspirin with placebo in patients treated with warfarin after heart-valve replacement. *N Engl J Med* 1993; 329: 524–529.
7 Mok CK, Boey J, Wang R et al. Warfarin versus dipyridamole-aspirin and pentoxifylline-aspirin for the prevention of prosthetic heart valve thromboembolism: a prospective randomized clinical trial. *Circulation* 1985; 72: 1059–1063.
8 Chesebro JH, Fuster V, Elveback LR et al. Trial of combined warfarin plus dipyridamole or aspirin therapy in prosthetic heart valve replacement: danger of aspirin compared with dipyridamole. *Am J Cardiol* 1983; 51: 1537–1541.
9 Koudstaal PJ. Antiplatelet therapy for preventing stroke in patients with nonrheumatic atrial fibrillation and a history of stroke or transient ischemic attacks. *Cochrane Database Syst Rev* 2000; 2.
10 Benavente O, Hart R, Koudstaal P, Laupacis A, McBride R. Antiplatelet therapy for preventing stroke in patients with non-valvular atrial fibrillation and no previous history of stroke or transient ischemic attacks. *Cochrane Database Syst Rev* 2000; 2.
11 Murray CJ, Lopez AD. Mortality by cause for eight regions of the world: Global Burden of Disease Study. *Lancet* 1997; 349: 1269–1276.
12 Murray CJ, Lopez AD. Global mortality, disability, and the contribution of risk factors: global burden of disease study. *Lancet* 1997; 349: 1436–1442.
13 Murray CJ, Lopez AD. Alternative projections of mortality and disability by cause 1990–2020: global burden of disease study. *Lancet* 1997; 349: 1498–1504.
14 Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes (2). *N Engl J Med* 1992; 326: 310–318.
15 Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes (1). *N Engl J Med* 1992; 326: 242–250.
16 Vorchheimer DA, Badimon JJ, Fuster V. Platelet glycoprotein IIb/IIIa receptor antagonists in cardiovascular disease. *J Am Med Assn* 1999; 281: 1407–1414.
17 Awtry EH, Loscalzo J. Aspirin. *Circulation* 2000; 101: 1206–1218.
18 Sharis PJ, Cannon CP, Loscalzo J. The antiplatelet effects of ticlopidine and clopidogrel. *Ann Intern Med* 1998; 129: 394–405.
19 Topol EJ, Byzova TV, Plow EF. Platelet GPIIb–IIIa blockers. *Lancet* 1999; 353: 227–231.
20 Peto R, Gray R, Collins R et al. Randomised trial of prophylactic daily aspirin in British male doctors. *Br Med J* 1988; 296: 313–316.
21 Final report on the aspirin component of the ongoing Physicians' Health Study. Steering Committee of the Physicians' Health Study Research Group. *N Engl J Med* 1989; 321: 129–135.
22 Thrombosis prevention trial: randomised trial of low-intensity oral anticoagulation with warfarin and low-dose aspirin in the primary prevention of ischaemic heart disease in men at increased risk. The Medical Research Council's General Practice Research Framework. *Lancet* 1998; 351: 233–241.
23 Hansson L, Zanchetti A, Carruthers SG et al. Effects of intensive blood-pressure lowering and low-dose aspirin in patients with hypertension: principal results of the Hypertension Optimal Treatment (HOT) randomised trial. HOT Study Group. *Lancet* 1998; 351: 1755–1762.
24 Hebert PR, Hennekens CH. An overview of the 4 randomized trials of aspirin therapy in the primary prevention of vascular disease. *Arch Intern Med* 2000; 160: 3123–3127.
25 The aspirin myocardial infarction study: final results. The Aspirin Myocardial Infarction Study research group. *Circulation* 1980; 62: V79–V84.
26 Aspirin in coronary heart disease. The Coronary Drug Project Research Group. *J Chronic Dis* 1976; 29: 625–642.
27 Elwood PC, Cochrane AL, Burr ML et al. A randomized controlled trial of acetyl salicylic acid in the secondary prevention of mortality from myocardial infarction. *Br Med J* 1974; 1: 436–440.
28 Persantine and aspirin in coronary heart disease. The Persantine- Aspirin Reinfarction Study Research Group. *Circulation* 1980; 62: 449–461.
29 Elwood PC, Sweetnam PM. Aspirin and secondary mortality after myocardial infarction. *Lancet* 1979; 2: 1313–1315.
30 Breddin K, Loew D, Lechner K, Oberla K, Walter E. The German-Austrian aspirin trial: a comparison of acetylsalicylic acid, placebo and phenprocoumon in secondary prevention of myocardial infarction. On behalf of the German-Austrian Study Group. *Circulation* 1980; 62: V63–72.
31 Collaborative overview of randomised trials of antiplatelet therapy – I: Prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. Antiplatelet Trialists' Collaboration. *Br Med J* 1994; 308: 81–106.
32 Janzon L, Bergqvist D, Boberg J et al. Prevention of myocardial infarction and stroke in patients with intermittent claudication; effects of ticlopidine. Results from STIMS, the Swedish Ticlopidine Multicentre Study. *J Intern Med* 1990; 227: 301–308.
33 A randomised, blinded, trial of clopidogrel versus aspirin in

Cambridge Books Online © Cambridge University Press, 2009

patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. *Lancet* 1996; 348: 1329–1339.

34 Rupprecht HJ. Consistency of the benefit of clopidogrel across a range of vascular-related endpoints: results from CAPRIE [Abstract]. *Eur Heart J* 1998; 19 (Suppl.): 52.

35 Ault KA, Cannon CP, Mitchell J et al. Platelet activation in patients after an acute coronary syndrome: results from the TIMI-12 trial. Thrombolysis in Myocardial Infarction. *J Am Coll Cardiol* 1999; 33: 634–639.

36 Cannon CP, McCabe CH, Borzak S et al. Randomized trial of an oral platelet glycoprotein IIb/IIIa antagonist, sibrafiban, in patients after an acute coronary syndrome: results of the TIMI 12 trial. Thrombolysis in Myocardial Infarction. *Circulation* 1998; 97: 340–349.

37 Cannon CP, McCabe CH, Wilcox RG et al. Oral glycoprotein IIb/IIIa inhibition with orbofiban in patients with unstable coronary syndromes (OPUS-TIMI 16) trial. *Circulation* 2000; 102: 149–156.

38 Kereiakes DJ, Kleiman NS, Ferguson JJ et al. Pharmacodynamic efficacy, clinical safety, and outcomes after prolonged platelet Glycoprotein IIb/IIIa receptor blockade with oral xemilofiban: results of a multicenter, placebo-controlled, randomized trial. *Circulation* 1998; 98: 1268–1278.

39 Dooley M, Goa KL. Sibrafiban. *Drugs* 1999; 57: 225–230.

40 O'Neill WW, Serruys P, Knudtson M et al. Long-term treatment with a platelet glycoprotein-receptor antagonist after percutaneous coronary revascularization. EXCITE Trial Investigators. Evaluation of Oral Xemilofiban in Controlling Thrombotic Events. *N Engl J Med* 2000; 342: 1316–1324.

41 Investigators. S. Comparison of sibrafiban with aspirin for prevention of cardiovascular events after acute coronary syndromes: a randomised trial. The SYMPHONY Investigators. Sibrafiban versus aspirin to yield maximum protection from ischemic heart events post-acute coronary syndromes. *Lancet* 2000; 355: 337–345.

42 Newby LK. Long-term oral platelet glycoprotein IIb/IIIa receptor antagonism with sibrafiban after acute coronary syndromes: study design of the sibrafiban versus aspirin to yield maximum protection from ischemic heart events post-acute coronary syndromes (SYMPHONY) trial. Symphony Steering Committee. *Am Heart J* 1999; 138: 210–218.

43 Chew DP, Bhatt DL, Sapp S, Topol EJ. Increased mortality with oral platelet glycoprotein IIb/IIIa antagonists: a meta-analysis of the phase III multi-center randomized trials. *Circulation* [in press] 2001.

44 Ferguson JJ. Meeting highlights. Highlights of the 48th scientific sessions of the American College of Cardiology. *Circulation* 1999; 100: 570–575.

45 Akkerhuis KM. Six month results of the FROST trial. Presented at the XXII Congress of the European Society of Cardiology. Amsterdam, Holland, 2000.

46 Akkerhuis M, Roth UJH, Brickl R, Simoons ML. Level of inhibition of platelet aggregation predicts outcome of patients treated with the oral glycoprotein IIb/IIIa receptor antagonist lefradafiban: Results from FROST [abstract]. *Circulation* 2000; 102 (Suppl II): II-377.

47 Muller TH, Weisenberger H, Brickl R, Narjes H, Himmelsbach F, Krause J. Profound and sustained inhibition of platelet aggregation by Fradafiban, a nonpeptide platelet glycoprotein IIb/IIIa antagonist, and its orally active prodrug, Lefradafiban, in men. *Circulation* 1997; 96: 1130–1138.

48 Guth BD, Seewaldt-Becker E, Himmelsbach F, Weisenberger H, Muller TH. Antagonism of the GPIIb/IIIa receptor with the nonpeptidic molecule BIBU52: inhibition of platelet aggregation in vitro and antithrombotic efficacy in vivo. *J Cardiovasc Pharmacol* 1997; 30: 261–272.

49 Akkerhuis KM, Neuhaus KL, Wilcox RG et al. Safety and preliminary efficacy of one month glycoprotein IIb/IIIa inhibition with lefradafiban in patients with acute coronary syndromes without ST-elevation A phase II study. *Eur Heart J* 2000; 21: 2042–2055.

50 Mousa SA, Bozarth J, Youssef A, Levine B. Oral antiplatelet efficacy of the platelet GPIIb/IIIa antagonist, DMP754 in non-human primates. *Thromb Res* 1998; 89: 217–225.

51 Mousa SA, Forsythe M, Lorelli W et al. Novel nonpeptide antiplatelet glycoprotein IIb/IIIa receptor antagonist, DMP754: receptor binding affinity and specificity. *Coron Artery Dis* 1996; 7: 767–774.

52 Harrington RA, Armstrong PW, Graffagnino C et al. Dose-finding, safety, and tolerability study of an oral platelet glycoprotein IIb/IIIa inhibitor, lotrafiban, in patients with coronary or cerebral atherosclerotic disease. *Circulation* 2000; 102: 728–735.

53 Topol EJ, Easton JD, Amarenco P et al. Design of the blockade of the glycoprotein IIb/IIIa receptor to avoid vascular occlusion (BRAVO) trial. *Am Heart J* 2000; 139: 927–933.

54 Lewis HD, Jr., Davis JW, Archibald DG et al. Protective effects of aspirin against acute myocardial infarction and death in men with unstable angina. Results of a Veterans Administration Cooperative Study. *N Engl J Med* 1983; 309: 396–403.

55 Theroux P, Ouimet H, McCans J et al. Aspirin, heparin, or both to treat acute unstable angina. *N Engl J Med* 1988; 319: 1105–1111.

56 The RISC Group. Risk of myocardial infarction and death during treatment with low dose aspirin and intravenous heparin in men with unstable coronary artery disease. *Lancet* 1990; 336: 827–830.

57 Wallentin LC. Aspirin (75 mg/day) after an episode of unstable coronary artery disease: long-term effects on the risk for myocardial infarction, occurrence of severe angina and the need for revascularization. Research Group on Instability in Coronary Artery Disease in Southeast Sweden. *J Am Coll Cardiol* 1991; 18: 1587–1593.

58 Cairns JA, Gent M, Singer J et al. Aspirin, sulfinpyrazone, or both in unstable angina. Results of a Canadian multicenter trial. *N Engl J Med* 1985; 313: 1369–1375.

59 Scrutinio D, Lagioia R, Rizzon P. Ticlopidine treatment for patients with unstable angina at rest. A further analysis of the

Cambridge Books Online © Cambridge University Press, 2009

study of ticlopidine in unstable angina. Studio della Ticlopidina nell'Angina Instabile Group. *Eur Heart J* 1991; 12 Suppl G: 27–29.
60 Balsano F, Rizzon P, Violi F et al. Antiplatelet treatment with ticlopidine in unstable angina. A controlled multicenter clinical trial. The Studio della Ticlopidina nell'Angina Instabile Group. *Circulation* 1990; 82: 17–26.
61 The Clopidogrel in Unstable angina to prevent Recurrent Events (CURE) trial programme. Rationale, design and baseline characteristics including a meta-analysis of the effects of thienopyridines in vascular disease. *Eur Heart J* 2000; 21: 2033–2041.
62 Theroux P, White H, David D et al. A heparin-controlled study of MK-383 in unstable angina [abstract]. *Circulation* 1994; 90 (Suppl I): I-231.
63 Theroux P, Kouz S, Roy L et al. Platelet membrane receptor glycoprotein IIb/IIIa antagonism in unstable angina. The Canadian Lamifiban Study. *Circulation* 1996; 94: 899–905.
64 Schulman SP, Goldschmidt-Clermont PJ, Topol EJ et al. Effects of integrelin, a platelet glycoprotein IIb/IIIa receptor antagonist, in unstable angina. A randomized multicenter trial. *Circulation* 1996; 94: 2083–2089.
65 Randomised placebo-controlled and balloon-angioplasty-controlled trial to assess safety of coronary stenting with use of platelet glycoprotein-IIb/IIIa blockade. The EPISTENT Investigators. Evaluation of Platelet IIb/IIIa Inhibitor for Stenting. *Lancet* 1998; 352: 87–92.
66 Lefkovits J, Ivanhoe RJ, Califf RM et al. Effects of platelet glycoprotein IIb/IIIa receptor blockade by a chimeric monoclonal antibody (abciximab) on acute and six-month outcomes after percutaneous transluminal coronary angioplasty for acute myocardial infarction. EPIC investigators. *Am J Cardiol* 1996; 77: 1045–1051.
67 Randomised placebo-controlled trial of abciximab before and during coronary intervention in refractory unstable angina: the CAPTURE Study. *Lancet* 1997; 349: 1429–1435.
68 A comparison of aspirin plus tirofiban with aspirin plus heparin for unstable angina. Platelet Receptor Inhibition in Ischemic Syndrome Management (PRISM) Study Investigators. *N Engl J Med* 1998; 338: 1498–1505.
69 Heeschen C, Hamm CW, Goldmann B, Deu A, Langenbrink L, White HD. Troponin concentrations for stratification of patients with acute coronary syndromes in relation to therapeutic efficacy of tirofiban. PRISM Study Investigators. Platelet Receptor Inhibition in Ischemic Syndrome Management. *Lancet* 1999; 354: 1757–1762.
70 Inhibition of the platelet glycoprotein IIb/IIIa receptor with tirofiban in unstable angina and non-Q-wave myocardial infarction. Platelet Receptor Inhibition in Ischemic Syndrome Management in Patients Limited by Unstable Signs and Symptoms (PRISM-PLUS) Study Investigators.
71 International, randomized, controlled trial of lamifiban (a platelet glycoprotein IIb/IIIa inhibitor), heparin, or both in unstable angina. The PARAGON Investigators. Platelet IIb/IIIa Antagonism for the Reduction of Acute coronary syndrome events in a Global Organization Network. *Circulation* 1998; 97: 2386–2395.
72 Moliterno DJ, White HD. Unstable angina: PARAGON, PURSUIT, PRISM and PRISM-PLUS. In: Lincoff AM, Topol EJ, eds. *Contemporary cardiology: platelet glycoprotein IIb/IIIa inhibitors in cardiovascular disease*. Totawa, NJ: Humana Press; 1999: 201–227.
73 Moliterno DJ. Patient-specific dosing of IIb/IIIa antagonists during acute coronary syndromes: rationale and design of the PARAGON B study. The PARAGON B International Steering Committee. *Am Heart J* 2000; 139: 563–566.
74 Newby LK, Christenson RH, Ohman EM et al. Conversion of high-risk acute coronary syndromes to low(er) risk via the use of troponin and platelet glycoprotein IIb/IIIa blockade [abstract]. *Circulation* 2000; 102: II-589.
75 Lincoff AM, Harrington RA, Califf RM et al. Management of patients with acute coronary syndromes in the United States by platelet glycoprotein IIb/IIIa inhibition. Insights from the platelet glycoprotein IIb/IIIa in unstable angina: receptor suppression using integrilin therapy (PURSUIT) trial. *Circulation* 2000; 102: 1093–1100.
76 Kleiman NS, Lincoff AM, Flaker GC et al. Early percutaneous coronary intervention, platelet inhibition with eptifibatide, and clinical outcomes in patients with acute coronary syndromes. PURSUIT Investigators. *Circulation* 2000; 101: 751–757.
77 Bhatt DL, Topol EJ. Current role of platelet glycoprotein IIb/IIIa inhibitors in acute coronary syndromes. *J Am Med Assn* 2000; 284: 1549–1558.
78 Randomised trial of intravenous streptokinase, oral aspirin, both, or neither among 17,187 cases of suspected acute myocardial infarction: ISIS-2. ISIS-2 (Second International Study of Infarct Survival) Collaborative Group. *Lancet* 1988; 2: 349–360.
79 Baigent C, Collins R, Appleby P, Parish S, Sleight P, Peto R. ISIS-2: 10 year survival among patients with suspected acute myocardial infarction in randomised comparison of intravenous streptokinase, oral aspirin, both, or neither. The ISIS-2 (Second International Study of Infarct Survival) Collaborative Group. *Br Med J* 1998; 316: 1337–1343.
80 Brener SJ, Barr LA, Burchenal JE et al. Randomized, placebo-controlled trial of platelet glycoprotein IIb/IIIa blockade with primary angioplasty for acute myocardial infarction. ReoPro and Primary PTCA Organization and Randomized Trial (RAPPORT) Investigators. *Circulation* 1998; 98: 734–741.
81 Neumann FJ, Blasini R, Schmitt C et al. Effect of glycoprotein IIb/IIIa receptor blockade on recovery of coronary flow and left ventricular function after the placement of coronary-artery stents in acute myocardial infarction. *Circulation* 1998; 98: 2695–2701.
82 Morris DC. Results from late-breaking clinical trials sessions at ACCIS '99 and ACC '99. American College of Cardiology. *J Am Coll Cardiol* 1999; 34: 1–8.
83 Stone GW. Stenting and IIb/IIIa Receptor Blockade in Acute Myocardial Infarction: An Introduction to the CADILLAC Trial. *J Invasive Cardiol* 1998; 10 Suppl B: 36B-47B.

Cambridge Books Online © Cambridge University Press, 2009

84 Schomig A, Kastrati A, Dirschinger J et al. Coronary Stenting plus Platelet Glycoprotein IIb/IIIa Blockade Compared with Tissue Plasminogen Activator in Acute Myocardial Infarction. *N Engl J Med* 2000; 343: 385–391.
85 Kleiman NS, Ohman EM, Califf RM et al. Profound inhibition of platelet aggregation with monoclonal antibody 7E3 Fab after thrombolytic therapy. Results of the Thrombolysis and Angioplasty in Myocardial Infarction (TAMI) 8 Pilot Study. *J Am Coll Cardiol* 1993; 22: 381–389.
86 Ohman EM, Kleiman NS, Gacioch G et al. Combined accelerated tissue-plasminogen activator and platelet glycoprotein IIb/IIIa integrin receptor blockade with Integrilin in acute myocardial infarction. Results of a randomized, placebo-controlled, dose-ranging trial. IMPACT-AMI Investigators. *Circulation* 1997; 95: 846–854.
87 Investigators P. Combining thrombolysis with the platelet glycoprotein IIb/IIIa inhibitor lamifiban: results of the Platelet Aggregation Receptor Antagonist Dose Investigation and Reperfusion Gain in Myocardial Infarction (PARADIGM) trial. *J Am Coll Cardiol* 1998; 32: 2003–2010.
88 Chesebro JH, Knatterud G, Roberts R et al. Thrombolysis in Myocardial Infarction (TIMI) Trial, Phase I: A comparison between intravenous tissue plasminogen activator and intravenous streptokinase. Clinical findings through hospital discharge. *Circulation* 1987; 76: 142–154.
89 Antman EM, Giugliano RP, Gibson CM et al. Abciximab facilitates the rate and extent of thrombolysis: results of the thrombolysis in myocardial infarction (TIMI) 14 trial. The TIMI 14 Investigators. *Circulation* 1999; 99: 2720–2732.
90 Bode C, Smalling RW, Berg G et al. Randomized comparison of coronary thrombolysis achieved with double- bolus reteplase (recombinant plasminogen activator) and front-loaded, accelerated alteplase (recombinant tissue plasminogen activator) in patients with acute myocardial infarction. The RAPID II Investigators. *Circulation* 1996; 94: 891–898.
91 Schwartz L, Bourassa MG, Lesperance J et al. Aspirin and dipyridamole in the prevention of restenosis after percutaneous transluminal coronary angioplasty. *N Engl J Med* 1988; 318: 1714–1719.
92 Investigators B. A comparison of balloon-expandable-stent implantation with balloon angioplasty in patients with coronary artery disease. *N Engl J Med* 1994; 331: 489–495.
93 Investigators S. A randomized comparison of coronary stent placement and balloon angioplasty in the treatment of coronary artery disease. *N Engl J Med* 1994; 331: 496–501.
94 Hall P, Nakamura S, Maiello L et al. A randomized comparison of combined ticlopidine and aspirin therapy versus aspirin therapy alone after successful intravascular ultrasound-guided stent implantation. *Circulation* 1996; 93: 215–222.
95 Leon MB, Baim DS, Popma JJ et al. A clinical trial comparing three antithrombotic-drug regimens after coronary-artery stenting. Stent Anticoagulation Restenosis Study Investigators. *N Engl J Med* 1998; 339: 1665–1671.
96 Bertrand ME, Allain H, Lablanche JM. Results of a randomized trial of ticlopidine versus placebo for prevention of acute closure and restenosis after coronary angioplasty. The TACT study [abstract]. *Circulation* 1990; 82 (Suppl. III): III-90.
97 Schomig A, Neumann FJ, Kastrati A et al. A randomized comparison of antiplatelet and anticoagulant therapy after the placement of coronary-artery stents. *N Engl J Med* 1996; 334: 1084–1089.
98 Bertrand ME, Legrand V, Boland J et al. Randomized multicenter comparison of conventional anticoagulation versus antiplatelet therapy in unplanned and elective coronary stenting. The full anticoagulation versus aspirin and ticlopidine (FANTASTIC) study. *Circulation* 1998; 98: 1597–1603.
99 Urban P, Macaya C, Rupprecht HJ et al. Randomized evaluation of anticoagulation versus antiplatelet therapy after coronary stent implantation in high-risk patients: the multicenter aspirin and ticlopidine trial after intracoronary stenting (MATTIS). *Circulation* 1998; 98: 2126–2132.
100 Steinhubl SR, Lauer MS, Mukherjee DP et al. The duration of pretreatment with ticlopidine prior to stenting is associated with the risk of procedure-related non-Q-wave myocardial infarctions. *J Am Coll Cardiol* 1998; 32: 1366–1370.
101 Muller C, Buttner HJ, Petersen J, Roskamm H. A randomized comparison of clopidogrel and aspirin versus ticlopidine and aspirin after the placement of coronary-artery stents. *Circulation* 2000; 101: 590–593.
102 Bertrand ME, Rupprecht HJ, Urban P, Gershlick AH, Investigators f. Double-blind study of the safety of clopidogrel with and without a loading dose in combination with aspirin compared with ticlopidine in combination with aspirin after coronary stenting: The Clopidogrel Aspirin Stent International Cooperative Study (CLASSICS). *Circulation* 2000; 102: 624–629.
103 Taniuchi M, Kurz HI, Smith SC, Lasala JM. Ticlid or plavix post-stent (TOPPS): randomization to 2-week treatment [abstract]. *Circulation* 1999; 100 (Suppl I): I-379.
104 Berger PB, Bell MR, Rihal CS et al. Clopidogrel versus ticlopidine after intracoronary stent placement. *J Am Coll Cardiol* 1999; 34: 1891–1894.
105 Moussa I, Oetgen M, Roubin G et al. Effectiveness of clopidogrel and aspirin versus ticlopidine and aspirin in preventing stent thrombosis after coronary stent implantation. *Circulation* 1999; 99: 2364–2366.
106 Mehran R, Dangas G. Ticlopidine versus clopidogrel in patients receiving coronary stents. Transcatheter Therapeutics Conference, Washington, DC, 1999.
107 L'Allier PL, Aronow HD, Yadav JS, Schneider JP, Topol EJ, Ellis SG. Is clopidogrel a safe and effective adjunctive anti-platelet therapy for coronary artery stenting [abstract]. *J Am Coll Cardiol* 2000; 35 (Suppl. A): 66A.
108 Mishkel GJ, Aguirre FV, Ligon RW, Rocha-Singh KJ, Lucore CL. Clopidogrel as adjunctive antiplatelet therapy during coronary stenting. *J Am Coll Cardiol* 1999; 34: 1884–1890.
109 Plucinski DA, Scheltema K, Krusmark J, Panchyshyn N. A comparison of clopidogrel to ticlopidine therapy for the prevention of major adverse cardiac events at thirty days and six

Cambridge Books Online © Cambridge University Press, 2009

months following coronary stent implantation [abstract]. *J Am Coll Cardiol* 2000; 35 (Suppl. A): 67A.

110 Calver AL, Blows LJ, Harmer S et al. Clopidogrel for prevention of major cardiac events after coronary stent implantation: 30-day and 6-month results in patients with smaller stents. *Am Heart J* 2000; 140: 483–491.

111 Bhatt DL, Bertrand ME, Berger PB et al. Reduction in major adverse cardiac events, including mortality, after stenting using clopidogrel instead of ticlopidine [abstract]. *Circulation* 2000; 102 (Suppl II): II–XX.

112 Gasperetti CM, Gonias SL, Gimple LW, Powers ER. Platelet activation during coronary angioplasty in humans. *Circulation* 1993; 88: 2728–2734.

113 Pope CF, Ezekowitz MD, Smith EO et al. Detection of platelet deposition at the site of peripheral balloon angioplasty using indium-111 platelet scintigraphy. *Am J Cardiol* 1985; 55: 495–497.

114 Use of a monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high-risk coronary angioplasty. The EPIC Investigation. *N Engl J Med* 1994; 330: 956–961.

115 Topol EJ, Califf RM, Weisman HF et al. Randomised trial of coronary intervention with antibody against platelet IIb/IIIa integrin for reduction of clinical restenosis: results at six months. The EPIC Investigators. *Lancet* 1994; 343: 881–886.

116 Topol EJ, Ferguson JJ, Weisman HF et al. Long-term protection from myocardial ischemic events in a randomized trial of brief integrin beta3 blockade with percutaneous coronary intervention. EPIC Investigator Group. Evaluation of Platelet IIb/IIIa Inhibition for Prevention of Ischemic Complication. *J Am Med Assn* 1997; 278: 479–484.

117 Lincoff AM, Califf RM, Moliterno DJ et al. Complementary clinical benefits of coronary-artery stenting and blockade of platelet glycoprotein IIb/IIIa receptors. Evaluation of Platelet IIb/IIIa Inhibition in Stenting Investigators. *N Engl J Med* 1999; 341: 319–327.

118 Topol EJ, Mark DB, Lincoff AM et al. Outcomes at 1 year and economic implications of platelet glycoprotein IIb/IIIa blockade in patients undergoing coronary stenting: results from a multicentre randomised trial. EPISTENT Investigators. Evaluation of Platelet IIb/IIIa Inhibitor for Stenting. *Lancet* 1999; 354: 2019–2024.

119 Platelet glycoprotein IIb/IIIa receptor blockade and low-dose heparin during percutaneous coronary revascularization. The EPILOG Investigators. *N Engl J Med* 1997; 336: 1689–1696.

120 Marso SP, Lincoff AM, Ellis SG et al. Optimizing the percutaneous interventional outcomes for patients with diabetes mellitus: results of the EPISTENT (Evaluation of platelet IIb/IIIa inhibitor for stenting trial) diabetic substudy. *Circulation* 1999; 100: 2477–2484.

121 Acute platelet inhibition with abciximab does not reduce in-stent restenosis (ERASER study). The ERASER Investigators. *Circulation* 1999; 100: 799–806.

122 Neumann FJ, Kastrati A, Schmitt C et al. Effect of glycoprotein IIb/IIIa receptor blockade with abciximab on clinical and angiographic restenosis rate after the placement of coronary stents following acute myocardial infarction. *J Am Coll Cardiol* 2000; 35: 915–921.

123 Harrington RA, Kleiman NS, Kottke-Marchant K et al. Immediate and reversible platelet inhibition after intravenous administration of a peptide glycoprotein IIb/IIIa inhibitor during percutaneous coronary intervention. *Am J Cardiol* 1995; 76: 1222–1227.

124 Tcheng JE, Harrington RA, Kottke-Marchant K et al. Multicenter, randomized, double-blind, placebo-controlled trial of the platelet integrin glycoprotein IIb/IIIa blocker Integrilin in elective coronary intervention. IMPACT Investigators. *Circulation* 1995; 91: 2151–2157.

125 Randomised placebo-controlled trial of effect of eptifibatide on complications of percutaneous coronary intervention: IMPACT-II. Integrilin to Minimise Platelet Aggregation and Coronary Thrombosis-II. *Lancet* 1997; 349: 1422–1428.

126 Gibson CM, Cohen DJ, Cohen EA et al. Eptifibatide improves coronary flow reserve following stent placement: an ESPRIT substudy [abstract]. *Circulation* 2000; 102 (Suppl II): II-366.

127 Investigators R. Effects of platelet glycoprotein IIb/IIIa blockade with tirofiban on adverse cardiac events in patients with unstable angina or acute myocardial infarction undergoing coronary angioplasty. The RESTORE Investigators. Randomized Efficacy Study of Tirofiban for Outcomes and REstenosis. *Circulation* 1997; 96: 1445–1453.

128 Gibson CM, Goel M, Cohen DJ et al. Six-month angiographic and clinical follow-up of patients prospectively randomized to receive either tirofiban or placebo during angioplasty in the RESTORE trial. Randomized Efficacy Study of Tirofiban for Outcomes and Restenosis. *J Am Coll Cardiol* 1998; 32: 28–34.

129 Topol EJ. New Platelet Glycoprotein IIb/IIIa Inhibitor Trials in Percutaneous Coronary Intervention. *J Invasive Cardiol* 1999; 11 Suppl C: 3C–6C.

130 PPP Collaborative Group. Low-dose aspirin and vitamin E in people at cardiovascular risk: a randomised trial in general practice. Collaborative Group of the Primary Prevention Project. *Lancet* 2001; 357: 89–95.

131 Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. *N Engl J Med* 2001; 345: 494–502.

132 Cannon CP, Weintraub WS, Demopoulos LA, et al. Comparison of early invasive and conservative strategies in patients with unstable coronary syndromes treated with the glycoprotein IIb/IIIa inhibitor tirofiban. *N Engl J Med* 2001; 344: 1879–1887.

133 Topol EJ. Reperfusion therapy for acute myocardial infarction with fibrinolytic therapy or combination reduced fibrinolytic therapy and platelet glycoprotein IIb/IIIa inhibition: the GUSTO V randomised trial. *Lancet* 2001; 357: 1905–1914.

134 ASSENT-3 Investigators. Efficacy and safety of tenecteplase in combination with enoxaparin, abciximab, or unfractionated heparin: the ASSENT-3 randomised trial in acute myocardial infarction. *Lancet* 2001; 358: 605–613.

Cambridge Books Online © Cambridge University Press, 2009

135 Topol EJ, Moliterno DJ, Herrmann HC, et al. Comparison of two platelet glycoprotein IIb/IIIa inhibitors, tirofiban and abciximab, for the prevention of ischemic events with percutaneous coronary revascularization. *N Engl J Med* 2001; 344: 1888–1894.

136 Mehta SR, Yusuf S, Peters RJ, et al. Effects of pretreatment with clopidogrel and aspirin followed by long-term therapy in patients undergoing percutaneous coronary intervention: the PCI–CURE study. *Lancet* 2001; 358: 527–533.

Cambridge Books Online © Cambridge University Press, 2009

70

Antiplatelet therapies in neurology

Graeme J. Hankey

Royal Perth Hospital, Western Australia and Department of Medicine, University of Western Australia

The major role of antiplatelet therapy in neurology is in the acute treatment and secondary prevention of ischemic stroke caused by arterial thromboembolism and, in some patients, atrial fibrillation. Because ischemic stroke is one of the most common neurological conditions[1], antiplatelet agents are used widely by neurologists and their patients.

Stroke

Definition

Stroke is characterized by rapidly developing clinical symptoms and signs of focal, and at times global, loss of cerebral function lasting more than 24 hours or leading to death, with no apparent cause other than that of vascular origin[2,3].

Incidence/prevalence

Stroke is a worldwide problem with an incidence of about 2 per 1000 per year[1,4,5]. The prevalence is about 9–12 per 1000[1,6]. Stroke can occur at any age, but half of all strokes occur in people over 70–75 years of age.

Etiology

About 80% of all strokes are ischemic in nature and are due to cerebral infarction, usually resulting from thrombotic or embolic occlusion of a cerebral artery; the remainder are caused by either intracerebral hemorrhage (15%) or subarachnoid hemorrhage (5%)[7]. The causes of ischemic stroke include embolism via, or from, the heart (20%), large artery atherothromboembolism (45–50%), small artery microatheroma/lipohyalinosis (25%), other arteriopathies such as dissection and arteritis (5%), and hematological disorders causing a prothrombotic state (<5%)[3].

Prognosis

Stroke kills 4.5 million people each year throughout the world, making it the third most common cause of death in most developed countries[8]. About 10% of individuals with acute ischemic stroke die within 30 days of stroke onset, and 30% by 1 year[7]. Among those who survive the acute event, about half will experience some level of disability at 6 months after the stroke, making stroke one of the major causes of physical disability[9,10]. Despite best medical and surgical strategies of secondary stroke prevention, recurrent stroke occurs in about 10% of cases within the first year after stroke[11].

Pathogenesis of ischemic stroke

Acute cerebral ischemia begins with the occlusion of a cerebral blood vessel, usually by *in situ* thrombus or embolism of thrombus (or other material) from a more proximal source[12]. Modern ideas about the pathogenesis of vessel thrombosis began with Rudolph Virchow's dissertation in 1845 in which he enunciated his famous triad that thrombosis was due to changes in the vessel wall, changes in the pattern of blood flow and changes in the constituents of the blood. Indeed, this concept had been already hinted at by John Hunter nearly 100 years earlier[13]. However, it has since been established that the most critical event in the formation of an arterial thrombus is injury to the vascular endothelium[14,15].

Normal endothelium

Under resting physiological conditions, the endothelium prevents thrombus formation. It acts as a physical barrier separating hemostatic from reactive subendothelial components and its negative surface charge may help to repel platelets. It possesses anticoagulant properties attributable to constitutional expression of thrombomodulin and

heparan sulfate, endogenous synthesis of ectoenzymes which degrade platelet agonists such as ADP, and endogenous synthesis of high local concentrations of the vasodilators prostacyclin (PGI_2) and nitric oxide (NO) which inhibit platelet aggregation.

Atherosclerosis

The most common cause of injury to the vascular endothelium is atherosclerosis, but there are other causes such as arterial dissection and trauma. Atherosclerosis is a disseminated pathological process which affects large- and medium-sized arteries, particularly at branching points. The most commonly affected sites in the cerebral circulation are the aortic arch, the origin of the internal carotid artery (ICA) in Caucasians, the siphon of the intracranial ICA, the origin of the middle cerebral artery and the vertebral and basilar arteries.

Acute coronary syndromes, and presumably many acute ischemic stroke syndromes caused by arterial disease, are precipitated by thrombus which develops on an atherosclerotic plaque in which the overlying endothelium is eroded or, perhaps more commonly, the atherosclerotic plaque has ruptured[16–18].

The initiation, progression and maturation of atherosclerotic plaque have been well documented[15,19]. Furthermore, it is now realized that inflammation in the vessel wall plays an essential part in the initiation and progression of atherosclerosis as well as in the erosion or fissure of plaques and, eventually, in the rupture of plaques[15]. However, it remains uncertain exactly what triggers a 'dormant' atherosclerotic plaque to become active and symptomatic.

The vulnerable, unstable plaque

Ruptured plaques contain a large core of eccentrically located lipid-laden macrophages (foam cells) engorged with oxidized low density lipoprotein (LDL) cholesterol, and a thin friable overlying fibrous cap devoid of smooth muscle cells[16,18,20]. The oxidized LDL within the lipid core stimulates plaque inflammation, which undermines the structural integrity of the plaque and activates the endothelium to a proinflammatory and procoagulant state[21–23]. The oxidized LDL and other inflammatory stimuli initiate recruitment of inflammatory cells into the lesion, and serve as a second messenger to enhance the synthesis of other vascular inflammatory products such as adhesion molecules and cytokines. Cytokines and metalloproteinases weaken the fibrous cap. The plaque is believed to rupture because the large lipid core redistributes the shear stress on the thin fibrous cap and very high loads are imparted upon localized areas of the weakened cap[24].

Triggers of plaque rupture

Recent epidemiological studies suggest that plaque inflammation (and rupture) may be triggered by exposure (acute or chronic) to an exogenous infectious antigen. There is a consistent significant relationship between symptomatic coronary artery disease and moderately elevated markers of inflammation that are unrelated to chronic infection (e.g. fibrinogen, C-reactive protein, albumin, serum amyloid A, and leukocyte count), particularly C-reactive protein[25,26]. Some studies have identified a higher than expected incidence of chronic infections of the teeth, gums and lungs, and with *Chlamydia pneumoniae*, *Helicobacter pylori*, and cytomegalovirus[15,27–29]. Although there is emerging evidence that these associations with chronic infections may be coincidental rather than causal[30–32], a similar, yet less robust, body of evidence is also mounting for the role of a systemic, low grade, inflammatory response being an integral part of the pathogenesis of acute ischemic stroke due to atherosclerosis[33–40].

Sequelae of plaque rupture: atherothrombosis

Following plaque rupture or endothelial erosion, blood is exposed to the endothelial basement membrane and extracellular matrix. Von Willebrand factor (vWF), which is a large, multimeric protein synthesized by the endothelium and secreted into the subendothelium, binds to extracellular collagen, primarily through its A3 domain. Platelets adhere to the subendothelial collagen (particularly types I and III), von Willebrand factor and fibronectin by means of platelet–membrane glycoprotein receptors, which are receptors for adhesive proteins. The largest glycoprotein (Gp) is designated I, the smallest IX. Letters a and b were added when better techniques allowed resolution of single protein bands on electrophoresis into two separate bands (e.g. glycoprotein I became Ia and Ib)[41]. The most abundant receptor is the integrin family which are heterodimic molecules composed of α and β subunits[42].

Under conditions of low shear stress, platelets adhere to subendothelial collagen and fibronectin through the binding of platelet glycoprotein Ia–IIa receptors[41,43]. Under conditions of high shear stress, platelets adhere to subendothelial vWF by means of platelet glycoprotein Ib–V–IX (Ib/IX)[43].

Platelets become activated when specific platelet receptors bind to various agonists such as collagen, thrombin, thromboxane A2, adenosine diphosphate (ADP), adrenaline and arachidonic acid. A series of intracellular reactions takes place. The final common pathway of platelet activation is the assembly of the glycoprotein IIb/IIIa receptor on the surface of activated platelets. Under resting conditions,

Cambridge Books Online © Cambridge University Press, 2009

the surface of a platelet contains 50 000 to 80 000 copies of the Gp IIb–IIIa ($\alpha_{IIb}\beta_3$) receptor. Upon platelet activation, the platelet GPIIb–IIIa receptor undergoes a conformational change, enabling it to bind both vWF and fibrinogen, resulting in irreversible platelet adhesion and aggregation, respectively. The dimeric nature of the fibrinogen molecule allows it to bind to Gp IIb–IIIa receptors on two separate platelets, resulting in interplatelet bridging, platelet aggregation, and growth of the primary platelet clot[44,45].

Platelet recruitment into the thrombus

Adherent, activated platelets recruit additional platelets into the growing thrombus by three mechanisms which are coordinated around the central role of thrombin[43].

First, activated platelets release ADP from storage granules, and the ADP binds to the ADP receptor of adjacent platelets, which activates them.

Secondly, activated platelets generate and release arachidonic acid, which is metabolized by the enzyme cyclo-oxygenase to prostaglandin endoperoxide, which in turn is converted by thromboxane synthetase to thromboxane A_2, a potent vasoconstrictor and platelet agonist. The platelets also release other eicosanoids, such as prostaglandin F_{2alpha} and serotonin, which induce further vasoconstriction and platelet aggregation[46–49].

Third, the modified membrane of activated platelets promotes assembly of clotting factors on the platelet surface (V,X, VIII), thereby amplifying thrombin generation (see below)[50]. Thrombin is central to platelet aggregation.

Coagulation

Coagulation is initiated by exposure of blood to tissue factors located in the necrotic core of ruptured atherosclerotic plaques, in the subendothelium of injured vessels, and on the surface of activated leukocytes attracted to the damaged vessel. The original cascade/waterfall hypothesis of blood coagulation is that there are two activating pathways: (i) the tissue factor or extrinsic pathway; and (ii) the contact or intrinsic pathway. A revised hypothesis of blood coagulation maintains that there is a single coagulation pathway, triggered by vessel injury and tissue factor (TF) (the factor VIIa/TF complex)[51]. Tissue factor binds factor VIIa and the resulting factor VIIa tissue factor complex activates both factors IX and X (i.e. the intrinsic and extrinsic pathways are intergrated in vivo). Factor IXa assembles on the surface of activated platelets as part of the intrinsic tenase complex which comprises factor IXa, factor VIIIa and calcium. Factor Xa, generated through the extrinsic (factor VIIa/tissue factor) and the intrinsic tenase complex, assembles on the surface of activated platelets as part of the prothrombin-activating (prothrombinase) complex, which consists of factor Xa, factor Va and calcium. When assembled in this way, the prothrombinase complex generates a burst of thrombin activity[43]. Thrombin (IIa) activates platelets and factors V and VIII and also converts fibrinogen to fibrin; thrombin then binds to fibrin where it remains active.

As blood clot forms at the site of vessel injury, plasminogen, an inert circulating protein closely bound to the deposited fibrin, is slowly activated (by tissue plasminogen activator which has been activated by kallikrein) to form plasmin, which digests the fibrin clot to give fibrin degradation products. The net result is that platelets and later fibrin accumulate at, and are limited to, the site of vascular injury. The rest of the vasculature remains free of platelet and fibrin deposits because, in circulating blood, the tendency of the coagulation mechanism to be activated is counterbalanced by inhibitory factors in the blood such as antithrombin III (which inactivates factors IX, X, XI and XII). However, at the point of vessel injury, the activation of the coagulation mechanism is so powerful that the inhibitors are overwhelmed.

The three major inhibitory systems which modulate the coagulation pathway are the protein C anticoagulant pathway, tissue factor pathway inhibitor (TFPI), and antithrombin. Protein C is activated by the thrombin/thrombomodulin complex on the endothelial cell surface. When thrombin binds to thrombomodulin (an endothelial membrane protein) it undergoes a conformational change at its active site that converts it from a procoagulant enzyme to a potent activator of protein C. Activated protein C acts as an anticoagulant in the presence of protein S by proteolytic degradation and inactivation of factors Va and VIIIa[50,52]. TFPI binds and inactivates factors Xa and the TFPI/factor Xa complex and then inactivates factor VIIa within the factor VIIa/tissue factor complex. Antithrombin inactivates free thrombin and factor Xa, but these clotting enzymes are protected from inactivation by antithrombin when they are bound to fibrin and activated platelets, respectively[43].

Antiplatelet therapy to prevent atherothrombosis

The preceding section highlights the pivotal role of platelets in atherothrombosis. It also underpins the rationale for, and biological plausibility of, antiplatelet therapy as an effective therapeutic strategy to prevent atherothrombosis and its sequelae (e.g. ischemic stroke).

The most reliable evidence for the effectiveness and safety of antiplatelet therapy in the acute treatment and

Cambridge Books Online © Cambridge University Press, 2009

secondary prevention of stroke are systematic reviews of all published and unpublished randomized controlled trials (RCTs)[53,54].

Antiplatelet therapy in acute ischemic stroke

There have been nine randomized trials of antiplatelet therapy in a total of 41 848 patients with acute ischemic stroke[53,55–61]. The antiplatelet regimens that have been compared with control are: aspirin 300 mg daily by mouth (or per rectum if unable to swallow)[55,56], aspirin 160 mg daily by mouth or nasogastric tube[57,58], ticlopidine[59], aspirin plus dipyridamole, OKY 046 (thromboxane synthase inhibitor)[60], and Abciximab[61]. Three of the trials, comprising a total of 162 patients, remain unpublished[53]. Two of the published studies, the International Stroke Trial (IST)[56] and the Chinese Acute Stroke Trial (CAST)[57], contribute 98% of all the data and both examined aspirin in acute ischemic stroke.

Aspirin in acute ischemic stroke

The IST and CAST randomized 40 090 patients within 48 hours of symptom onset to receive either aspirin (300 mg daily in IST, 160 mg daily in CAST) or no aspirin for the first 2 to 4 weeks following an acute ischemic stroke[56,57,62].

Early outcomes

A combined analysis of the patients randomized in CAST and IST found that early aspirin use within the first two to four weeks of acute ischemic stroke was associated with a highly significant reduction of 7 (SD 1) per 1000 in the risk of recurrent ischemic stroke in hospital (320 [1.6%] aspirin vs. 457 [2.3%] control, $2P<0.000001$) and a less clearly significant reduction of 4 (SD 2) per 1000 in death without further stroke (5.0% vs. 5.4%, $2P=0.05$)[62].

Against these early benefits, early aspirin use was associated with an increase of 2 (SD 1) per 1000 in hemorrhagic stroke or hemorrhagic transformation of the original infarct (1.0% vs. 0.8%, $2P=0.07$) and no apparent effect on further stroke of unknown cause (0.9% vs. 0.9%). Aspirin also increased the risk of major extracranial hemorrhage during the treatment period by four events per 1000 patients treated (0.97% vs. 0.57%, odds ratio [OR]: 1.7, 95% confidence interval [CI]: 1.3 to 2.1). However, for patients not also receiving anticoagulants, the excess was only two hemorrhages per 1000 patients treated (i.e. 0.2% excess). The total short-term net effect of early aspirin use was a reduction of 9 (SD 3) per 1000 in the overall risk of further stroke or death in hospital (8.2% vs. 9.1%, $2P=0.001$)[62].

The proportional and absolute benefits of aspirin in acute ischemic stroke were similar in a wide range of patients irrespective of age, gender, delay between symptom onset and randomization, presence or absence of atrial fibrillation, conscious level, systolic blood pressure, stroke subtype, CT brain scan findings, or concomitant heparin use[62]. Among the 9000 patients (22%) randomized without a prior CT brain scan, aspirin appeared to be of net benefit with no unusual excess of hemorrhagic stroke; moreover, even among the 800 (2%) who had inadvertently been randomized after a hemorrhagic stroke, there was no evidence of net hazard (further stroke or death, 63 aspirin vs. 67 control)[62].

Later outcomes

Early aspirin therapy was associated with a 5% (95% CI 2% to 9%) reduction in the odds of death or dependency at the end of follow-up at 1 to 6 months after stroke, corresponding to an absolute reduction of 13 per 1000 patients treated[53].

Other antiplatelet agents in acute ischemic stroke

Limited, current data indicate that other antiplatelet agents (e.g. ADP receptor antagonists (ticlopidine), platelet glycoprotein IIb/IIIa receptor antagonists (e.g. abciximab), thromboxane synthase inhibitors (e.g. OKY 046), and combination therapies (e.g. aspirin combined with dipyridamole)) are safe in acute ischemic stroke[59–61], but further trials are needed to establish their safety and efficacy.

Antiplatelet therapy for the secondary prevention of stroke and other serious vascular events

The most recent systematic review of the evidence from RCTs of all antiplatelet therapies in secondary prevention of stroke and other vascular events to have been published was undertaken by the Antiplatelet Trialists' Collaboration (APT) and published in 1994[54]. An updated review was undertaken by the Antithrombotic Trialists' (ATT) Collaboration in September 1997, but the results await publication[63]. In this section I will present the results of all relevant clinical trials published since 1994 and incorporate them into previous data published by the APT. Hopefully, this will provide an updated systematic review which will be consistent with the results of the anticipated forthcoming publication by the ATT in 2001 or 2002.

The APT (and ATT) analysed the individual patient data

Cambridge Books Online © Cambridge University Press, 2009

from each trial according to the qualifying medical condition of the patient, the type of antiplatelet regimen and the type of outcome events recorded.

The qualifying medical condition of the patient was categorised as symptomatic vascular disease of the brain, heart of limbs (i.e. previous TIA or stroke, previous MI, previous PAD).

The primary outcome event was the composite of non-fatal stroke, non-fatal myocardial infarction (MI) and death from vascular causes. This was chosen because these events generally share the same underlying pathophysiology, pathogenesis and response to antiplatelet therapy. Moreover, they are all important to avoid from the patient's perspective and, by combining them as a composite outcome, the total number of outcome events and hence statistical power, is maximized.

Because the analysis showed that the effects of antiplatelet agents were qualitatively the same in each subgroup of patients with symptomatic vascular disease of the brain, heart and limbs (i.e. antiplatelet therapy was effective for all qualifying conditions), and differed only quantitatively (i.e. the magnitude of the favourable effect varied), this group of patients at high risk of future vascular events were analysed together in order to further maximize statistical power. This concept of 'lumping' different qualifying conditions and vascular outcomes together is valid, and more precise, if the underlying cause of the conditions and response to the therapy is similar, as it is in this case. I have therefore presented the results of the APT and subsequent published trials in all high-risk patients (e.g. angina, MI, stroke, TIA, peripheral vascular disease, recent vascular surgery), because the effects are qualitatively the same and the precision of the estimates (i.e. statistical power) is maximized. However, in recognition of the pathophysiological heterogeneity of stroke (i.e. perhaps only a small majority of all strokes are due to atherothromboembolism), I have also presented the results for TIA and stroke patients, which are compromised a little by smaller sample sizes, and hence the estimates have wider confidence intervals and less precision.

All antiplatelet agents

Benefits

Antiplatelet therapy, given to around 70 000 patients at high risk of vascular disease (i.e. symptomatic vascular disease of the brain, heart and limbs) for a month or more, is associated with a reduction in odds of a subsequent serious vascular event (stroke, MI or vascular death) by about a quarter (odds reduction 27%; 95% CI 24% to 31%; $2P < 0.00001$)[54].

The proportional effects are similar in the various categories of patients, including more than 11 700 with a prior (presumed ischemic) stroke or transient ischemic attack (TIA), amongst whom the odds reduction is about 21 + 3%. The absolute risk reduction is about 3.7% over about 3 years, which means that 37 vascular events (20 non-fatal strokes, nine non-fatal myocardial infarctions, and 11 vascular deaths) are prevented per 1000 patients treated with antiplatelet therapy for about 2–3 years, or 15 events prevented per year. The number of patients needed to treat (NNT) with antiplatelet therapy to prevent one serious vascular event each year is therefore about 83 (i.e. 1000/12). Antiplatelet therapy is also associated with a 16% (SD 6) reduction in the odds of all-cause mortality among patients with prior TIA or stroke.

Risks

The most important adverse effect of antiplatelet therapy is bleeding, particularly intracranial hemorrhage because it is frequently fatal or disabling. However, the risk of intracranial hemorrhage associated with antiplatelet therapy is small, and at most one or two per 1000 patients treated per year[54]. This excess risk is included in the overall estimate above of the effect of antiplatelet therapy on serious vascular events (i.e. stroke, MI or vascular death).

Antiplatelet therapy is also associated with a small but significant excess of non-fatal extracranial hemorrhage (mainly from the gastrointestinal tract) of one to two per 1000 patients treated per year but there is no clear excess of fatal extracranial hemorrhage.

Thus, the relatively large absolute reduction in serious vascular events (15 events per 1000 patients treated per year) clearly outweighs the smaller hazards (1–2 non-fatal extracranial hemorrhages per 1000 patients treated per year)[54].

Aspirin

Benefit

Aspirin, the most widely studied antiplatelet drug, reduces the odds of a serious vascular event by about one quarter among high risk patients (odds reduction 25% (SD 2); 95% CI 21% to 30%; $2P < 0.00001$)[54].

In 11 placebo-controlled RCTs of aspirin in more than 10 000 patients with prior TIA or ischemic stroke, aspirin reduced the odds of a serious vascular event by 17% + 4.4 ($2P = 0.00009$). This is a relative risk reduction (RRR) of 13% (95%CI 6% to 19%) and an absolute risk reduction (ARR) of 3% over about 3 years, or about 1% per year (Fig. 70.1)[64]. The number of patients with previous TIA or ischemic

Cambridge Books Online © Cambridge University Press, 2009

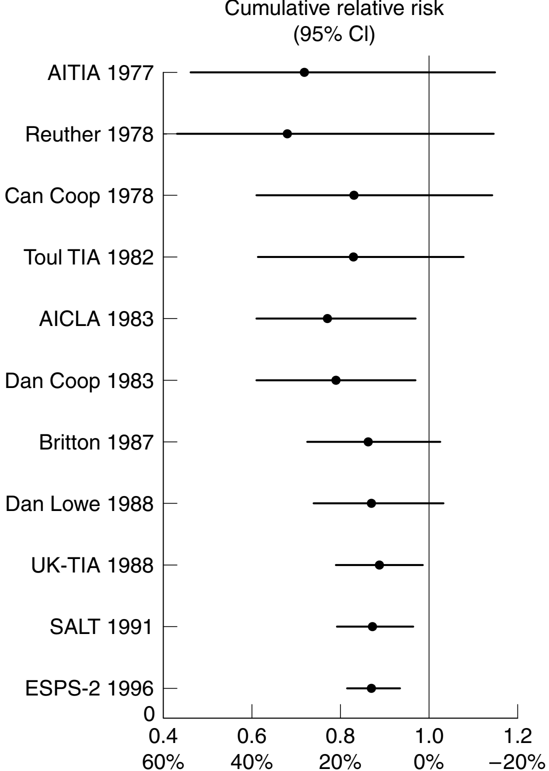


Fig. 70.1. Cumulative meta-analysis of 11 randomized controlled trials of aspirin vs. control in patients with previous TIA or ischemic stroke in chronological order (1977 to 1996), showing relative risks and corresponding relative risk reductions with 95% confidence intervals (CI). Each line represents the relative risk and 95% CI of that study combined with all previous studies[64].

stroke needed to treat with aspirin to prevent one serious vascular event each year (NNT) is therefore about 100.

Safety

Aspirin is associated with a dose-dependent increase in symptoms of upper gastrointestinal upset (nausea, heartburn and epigastric pain), upper GI bleeding, and intracranial hemorrhage, and a non-dose-dependent increase in constipation.

Observational studies reveal symptoms of upper gastrointestinal upset (nausea, heartburn, and epigastric pain) in about 15–32% of people taking placebo, about 30% of people taking aspirin 300 mg/day, and 24–44% of people taking 900–1300 mg aspirin per day[65]. Randomized trials involving direct comparisons of different doses of aspirin indicate that high dose (500–1500 mg daily) aspirin significantly increases the odds of upper gastrointestinal symptoms compared with medium dose (75–325 mg daily) aspirin (high dose 26.2%, medium dose 21.9%, OR 1.3, 95% CI 1.1 to 1.5)[66,67], and that medium dose (283 mg) aspirin is associated with a trend towards an increase in odds of upper gastrointestinal upset compared with low dose (30 mg) aspirin (medium dose 11.4%, low dose 10.5%, OR 1.1, 95% CI: 0.9 to 1.4)[68].

Observational and case-control studies reveal an increased rate of upper gastrointestinal (GI) and other extracranial hemorrhage in people taking aspirin, and probably also a dose–response[66–68]. The risk of hematemesis attributable to aspirin has been estimated to be 0.2 to 1.0 per 1000 person–years of exposure[69]. The odds ratio of GI bleeding is 2.3, 3.2 and 3.9 for 75 mg, 150 mg and 300 mg, respectively, on at least 5 days a week[70]. Direct comparisons of different doses of aspirin in randomized trials also indicate that high dose (500–1500 mg daily) aspirin is associated with a trend towards an increase in odds of upper GI hemorrhage compared with medium dose (75–325 mg daily) aspirin (high dose 2.6%, medium dose 1.9%, OR 1.4, 95% CI 0.9 to 2.1)[66,67], but there is no apparent increase in odds of upper GI hemorrhage between medium dose (283 mg) aspirin and low dose (30 mg) aspirin from the limited trial data available (medium dose 1.9%, low dose 1.8%, OR 1.2, 95% CI 0.7 to 2.0)[68]. The type of aspirin may also be important (as well as dose). At average daily doses of aspirin of 325 mg or less, the relative risks of upper GI hemorrhage for plain, enteric-coated, and buffered aspirin are about 2.6, 2.7, and 3.1, respectively[71]. At doses greater than 325 mg, the relative risk is about 5.8 for plain and 7.0 for buffered aspirin[71]. Indirect evidence suggests that enteric-coated aspirin is associated with less upper GI bleeding at doses above 325 mg/day[71].

Intracranial hemorrhage is also increased in people taking aspirin by about one per 1000, and there appears to be a dose–response[72].

Costs

Plain aspirin costs about Aus$ 20 per patient per year (Aus $1 = US$ 0.55 = Euro 0.6)[73], or about Aus $2000 to treat 100 TIA/stroke patients for one year to prevent one serious vascular event. The cost of 75 mg enteric-coated aspirin is more than 20 times that of 75 mg plain aspirin.

Different doses of aspirin

Controversy has surrounded the most appropriate dose of aspirin for the long-term secondary prevention of serious vascular events, particularly stroke, with arguments for the use of daily doses as low as 30 mg to as high as 1500 mg[74–77]. The rationale for the lower doses has been evidence from laboratory studies showing that single oral doses of 5 to 200 mg of aspirin result in dose-dependent inhibition of platelet cyclooxygenase activity, with 100 mg almost completely

Cambridge Books Online © Cambridge University Press, 2009

suppressing the biosynthesis of thromboxane A2[65]. Because the effect is permanent, lasting for the life of the platelet (8–10 days), the inhibitory effect of repeated daily doses below 100 mg is cumulative. Thus the daily administration of 30 to 50 mg aspirin results in complete suppression of platelet thromboxane biosynthesis after 7 to 10 days[78].

Benefit

The most reliable evidence of the relative effect of different doses of aspirin comes from direct comparisons in randomized trials. Direct comparisons of the effect of daily doses of 75–325 mg and 500–1500 mg among 2425 high-risk patients, two-thirds of whom had a history of ischemic stroke or TIA, showed that daily doses of 75–325 mg and 500–1500 mg were similarly effective[54,66]. A recent randomized trial comparing the effects of lower doses of aspirin (81 mg or 325 mg daily) and higher doses (650 mg or 1300 mg daily) among 2849 patients undergoing carotid endarterectomy found that the combined rate of stroke, myocardial infarction or death was lower in the low-dose groups than in the high-dose groups at 30 days (5.4 vs. 7.0%, $P=0.07$) and at 3 months follow-up (6.2% vs. 8.4%, $P=0.03$)[67]. In an efficacy analysis, which included patients taking 650 mg or more acetylsalicylic acid before randomization, and patients randomized within 1 day of carotid surgery, combined rates were 3.7% (low-dose) and 8.2% (high dose), respectively, at 30 days ($P=0.002$) and 4.2% (low dose) and 10.0% (high dose) at 3 months ($P=0.002$)[67]. The authors concluded that the risk of stroke, MI and death within 30 days and 3 months of carotid endarterectomy is lower for patients taking 81mg or 325 mg acetylsalicylic acid daily than for those taking 650 mg or 1300 mg daily[67].

A direct randomized comparison of the effects of daily doses of very low dose aspirin (30 mg) and medium dose aspirin (283 mg) among about 3000 patients with a recent TIA or minor ischemic stroke, found no significant difference in subsequent serious vascular events but the possibility of a small (yet clinically important) difference could not be excluded[68].

Indirect comparisons of the effect of different doses, as compared with placebo, are less reliable, in the same way that it is unreliable to compare different teams, athletes, or horses by their respective performances against an opponent in common; it is more reliable to have them oppose each other directly. Nevertheless, indirect comparisons of trials comparing daily doses of <160 mg (mostly 75–150 mg), 160–325 mg, and 500–1500 mg against placebo reveals similar proportional reductions in serious vascular events among those allocated low, medium and high-dose aspirin[54,79]. The inclusion of data from two large trials among high-risk patients (one of them including almost 1400 patients with a prior ischemic stroke or TIA) assessing a daily dose of 75 mg vs. control[80,81], showed that low-dose aspirin (75 mg daily) was associated with a clear reduction in vascular events (odds reduction 29%, 95% CI 17% to 39%), similar to the proportional effects of higher doses[54].

The effects of doses lower than 75 mg daily have been less extensively studied in randomized trials. In the second European Stroke Prevention Study (ESPS-2), around 6000 people with a prior stroke or TIA were randomized, in a factorial design, to receive aspirin 50 mg daily, modified release dipyridamole 400 mg daily, both or neither[82]. Compared with no antiplatelet therapy, aspirin 50 mg daily produced a reduction of 16% (95% CI 1% to 29%; $2P=0.02$) in the odds of stroke or death[82]. Since most non-fatal vascular outcomes in this group of patients were strokes, and most deaths were vascular, this outcome closely approximates vascular events.

The available evidence from randomized trials therefore suggests that aspirin at a dose of 75 mg daily is as effective as higher doses, but there is insufficient evidence to be certain that doses below 75 mg daily are as effective.

Alternative antiplatelet regimens to aspirin

In the last APT overview, neither direct nor indirect comparisons between different antiplatelet regimens provided clear evidence that any regimen was more effective than aspirin in the prevention of vascular events[54]. Direct comparisons of alternative antiplatelet regimens revealed that the most promising single agent was the thienopyridine derivative, ticlopidine; the three trials in which ticlopidine was compared directly with aspirin showed that ticlopidine was associated with a non-significant reduction in the odds of a vascular event of 10% (95% CI −1% to 24%) when compared directly with aspirin[54]. However, in comparison with aspirin, ticlopidine was associated with a threefold excess of neutropenia ($<1.2\times10^9$/litre) (2.3% ticlopidine vs. 0.8% aspirin; OR 2.7; 95% CI 1.5 to 4.8), and a twofold increase in the odds of skin rash (11.8% ticlopidine vs. 5.5% aspirin; OR 2.2; 95% CI 1.7 to 2.9) and of diarrhea (20.4% vs. 9.9%; OR 2.3; 95% CI 1.9 to 2.8)[54].

Two recent large trials have since provided further information about a new thienopyridine agent, clopidogrel[83], and the combination of aspirin and dipyridamole in patients with previous TIA or ischemic stroke[82].

The CAPRIE (Clopidogrel versus Aspirin in Patients at Risk of Ischemic Events) trial was designed to determine whether clopidogrel (a thienopyridine like ticlopidine) was as safe as aspirin and, like ticlopidine, also about 10% more effective than aspirin in preventing vascular events among patients with symptomatic atherothrombosis of the cere-

Cambridge Books Online © Cambridge University Press, 2009

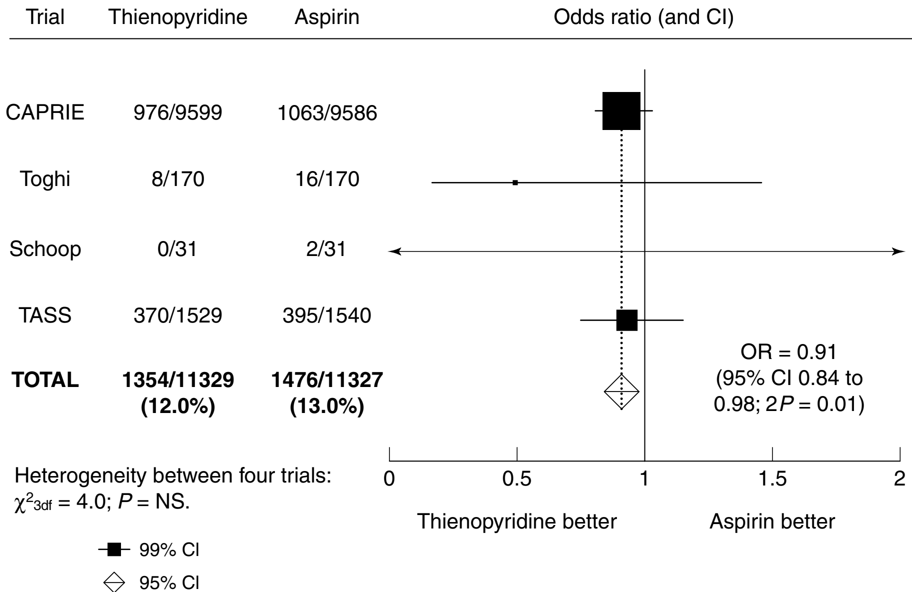


Fig. 70.2. Direct comparisons of the proportional effects of the thienopyridines vs. aspirin on vascular events (stroke, myocardial infarction, or vascular death) in patients at high risk of vascular disease. TASS: Ticlopidine Aspirin Stroke Study. CAPRIE: Clopidogrel vs. Aspirin in Patients at Risk of Ischemic Events[84].

bral, coronary and peripheral arteries[83]. The study was powered to detect a realistic (i.e. 10%) treatment effect in the whole cohort and not in the three clinical subgroups. Ultimately, 19 185 patients with recent ischemic stroke (6431), MI (6302), and PAD (6452) were randomized to 'gold standard' treatment with aspirin 325 mg daily or to clopidogrel 75 mg daily, over three years (mean 1.9 years)[83]. In CAPRIE, the annual risk of subsequent ischemic stroke, MI or vascular death (the primary outcome event) was 5.8% among patients allocated aspirin and 5.3% among patients allocated clopidogrel[83]. This is a relative risk reduction of 8.7% (95% CI 0.3% to 16.5%; $P=0.043$), and an absolute risk reduction of 0.51% per year. In CAPRIE, both drugs (aspirin and clopidogrel) had a low incidence and similar profile of adverse events; the only significant differences being a higher proportion of severe gastrointestinal (GI) hemorrhage in the aspirin group (0.49% vs. 0.71%) and skin rash in the clopidogrel group (0.26% vs. 0.10%). There was no significant difference in the occurrence of severe thrombocytopenia and neutropenia.

Thienopyridines (ticlopidine and clopidogrel)

A systematic review of the four randomized trials comparing the thienopyridines (either ticlopidine or clopidogrel) with aspirin among over 20 000 patients at high risk of vascular disease (including almost 10 000 presenting with a TIA or ischemic stroke) has since been undertaken[84].

Benefit

Compared with aspirin, allocation to a thienopyridine was associated with a modest, yet statistically significant, reduction in the odds of a serious vascular event (12.0% thienopyridine vs. 13.0% aspirin; OR 0.91; 95% CI 0.84 to 0.98; $2P=0.01$), corresponding to the prevention or delay of 11 (95% CI 2 to 19) vascular events per 1 000 patients treated for about 2 years (Fig. 70.2)[84].

The patients in the thienopyridine group also experienced a significant reduction in the odds of any stroke (5.7% thienopyridine vs. 6.4% aspirin; OR 0.88; 95% CI 0.79 to 0.98), corresponding to the prevention or delay of 7 (95% CI 1 to 13) strokes per 1000 patients treated for two years. There was also a non-significant trend towards a reduction in ischemic stroke (OR 0.90; 95% CI 0.81 to 1.01), myocardial infarction (OR 0.88; 95% CI 0.76 to 1.01), vascular or unknown cause of death (OR 0.93; 95% CI 0.82 to 1.06), and death from any cause (OR 0.95; 95% CI 0.85 to 1.05)[84].

Patients with stroke or TIA

Among the 9840 patients with TIA/ischemic stroke, the thienopyridines produced similar proportional benefits to those found overall, both in terms of vascular events (16.8% thienopyridine vs. 18.3% aspirin; OR 0.90; 95% CI 0.81 to 1.00) and in terms of any stroke (10.4% vs. 12.0%; OR 0.86; 95% CI 0.75 to 0.97). The absolute reduction of 14 (95% CI −1 to 29) vascular events per 1000 patients treated for about 2 years was similar to that observed among all

Cambridge Books Online © Cambridge University Press, 2009

high-risk patients. However, the risk of stroke among patients with a previous TIA or ischemic stroke in the aspirin group (12.0%) was almost twice as high as that for all high-risk patients (6.4%). The absolute reduction of 16 strokes (95% CI: 3 to 28) per 1000 patients over about 2 years was therefore about twice as large as that for all high-risk patients combined[84].

If the risk of stroke and other serious vascular events is about 6.0% per year with aspirin therapy, then clopidogrel could reduce it by 10%, to 5.4% per year, which is an ARR of 0.6% per year. The NNT is 166 (100/0.6) compared with aspirin.

Safety

There was no clear difference between the thienopyridines and aspirin in the odds of experiencing an intracranial hemorrhage (0.3% thienopyridine vs. 0.4% aspirin; OR 0.82; 95% CI 0.53 to 1.27) or an extracranial hemorrhage (8.8% thienopyridine vs. 8.9% aspirin; OR 1.00; 95% CI 0.91 to 1.09)[84].

The thienopyridines were, however, associated with a significant reduction in the odds of both gastrointestinal hemorrhage (1.8% thienopyridine vs. 2.5% aspirin; OR 0.71; 95% CI 0.59 to 0.86) and indigestion/nausea/vomiting (14.8% vs 17.1%; OR 0.84; 95% CI 0.78 to 0.90), and with an increased odds of diarrhea, and of skin rash (Fig. 70.3). There was substantial heterogeneity between the results for ticlopidine and clopidogrel both for diarrhea (χ^2_{1df}= 13.4; 2P=0.0003) and for skin rash (χ^2_{1df}=17.9; 2P= 0.00002) (Fig. 70.3). Hence, in comparison with aspirin, ticlopidine produced about a twofold increase in the odds of skin rash (11.8% ticlopidine vs. 5.5% aspirin; OR 2.2; 95% CI 1.7 to 2.9) and of diarrhea (20.4% vs. 9.9%; OR 2.3; 95% CI 1.9 to 2.8), whilst clopidogrel produced a smaller increase of about one-third in the odds of skin rash (6.0% clopidogrel vs. 4.6% aspirin; OR 1.3; 95% CI 1.2 to 1.5) and of diarrhea (4.5% vs. 3.4%; OR 1.3; 95% CI 1.2 to 1.6) (Fig. 70.3).

Ticlopidine was associated with an excess of neutropenia (<1.2×10^9/litre) (2.3% ticlopidine vs. 0.8% aspirin; OR 2.7; 95% CI 1.5 to 4.8), but clopidogrel was not (0.1% clopidogrel vs. 0.2% aspirin; OR 0.63; 95% CI 0.29 to 1.36; test for heterogeneity between results for ticlopidine and clopidogrel: χ^2_{1df}=8.9; 2P=0.003) (Fig. 70.3).

There were no published trial data available for the frequency of thrombocytopenia associated with ticlopidine compared with aspirin, but observational data from 43 332 patients undergoing ticlopidine therapy in association with coronary artery stenting indicate that ticlopidine is associated with a significant excess incidence of thrombotic thrombocytopenic purpura (TTP) of 0.02% (95% CI 0.009% to 0.04%)[85], compared with the estimated incidence of 0.00037% (standardized morbidity ratio 56 95% CI 26 to 107)[86].

Clopidogrel was not associated with any excess of thrombocytopenia (<100×10^9 platelets per litre) compared with aspirin (0.26% vs. 0.26%; OR 1.00; 95% CI 0.57 to 1.74) among the 9553 patients who were screened before being allocated clopidogrel in CAPRIE, and of whom 99.8% were followed up carefully for an average of about 1.9 years[83]. However, recent reports of 20 cases of TTP associated with clopidogrel use has raised concerns about the safety of clopidogrel[87,88], particularly because clopidogrel and ticlopidine are thienopyridine derivatives which share a similar chemical structure, mechanism of antiplatelet action (ADP receptor blockade) and therapeutic efficacy; and because a causal association between ticlopidine and TTP was not established until several years after ticlopidine was introduced into clinical practice. However, it remains uncertain whether the reported association between clopidogrel and TTP is coincidental or causal[89]. Follow-up studies are in progress.

The results for adverse effects among patients with TIA/ischemic stroke were similar to those for all patients combined.

Cost

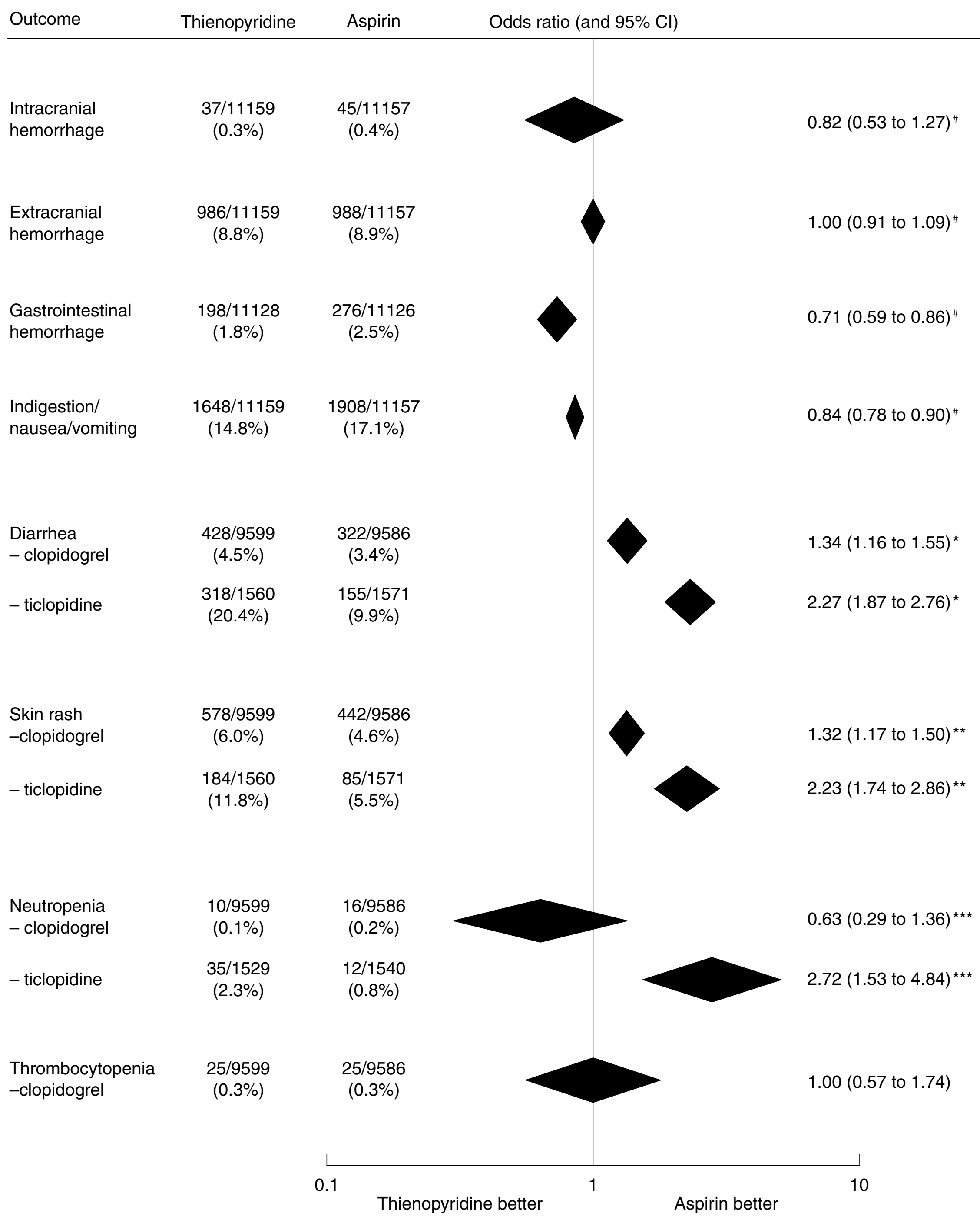
The cost of clopidogrel is Aus$ 1200 per patient per annum[73], or about Aus$ 200 000 to treat 166 TIA/stroke patients for 1 year to prevent one more serious vascular event than if they had been treated with aspirin.

Aspirin and dipyridamole

Benefit

In the APT overview, the addition of dipyridamole to aspirin among around 5000 high-risk patients was not associated with any significant reduction in vascular events, but the possibility of a small additional benefit was not excluded[54]. When data from a further 3000 patients in the second European Stroke Prevention Study (ESPS-2) study were included, the addition of dipyridamole to aspirin produced a non-significant reduction of 10% (95% CI 0% to 20%) in the odds of a vascular event (Fig. 70.4)[82,90]. Most of this reduction appeared to be attributable to a 23% reduction in non-fatal strokes, suggesting that the addition of dipyridamole to aspirin may be appropriate for patients at particularly high risk of stroke (Fig. 70.4)[90]. A detailed overview of all trials that have assessed the addition of dipyridamole to aspirin is currently being prepared by the Antithrombotic Trialists' Collaboration[63].

Cambridge Books Online © Cambridge University Press, 2009



No heterogeneity between trials
Heterogeneity between odds ratios for ticlopidine and clopidogrel: $\chi^2_{1df} = 17.9$; $2P = 0.00002$
* Heterogeneity between odds ratios for ticlopidine and clopidogrel: $\chi^2_{1df} = 13.4$; $2P = 0.0003$
** Heterogeneity between odds ratios for ticlopidine and clopidogrel: $\chi^2_{1df} = 8.9$; $2P = 0.003$

Diamonds representing the odds ration and 95% CI for the top (intracranial haemorrhage, etc) are for any thienopyridine (i.e. ticlopidine or clopidogrel.

Fig. 70.3. Direct comparisons of the proportional effects of the thienopyridines vs. aspirin on adverse events in patients at high risk of vascular disease[84].

Cambridge Books Online © Cambridge University Press, 2009

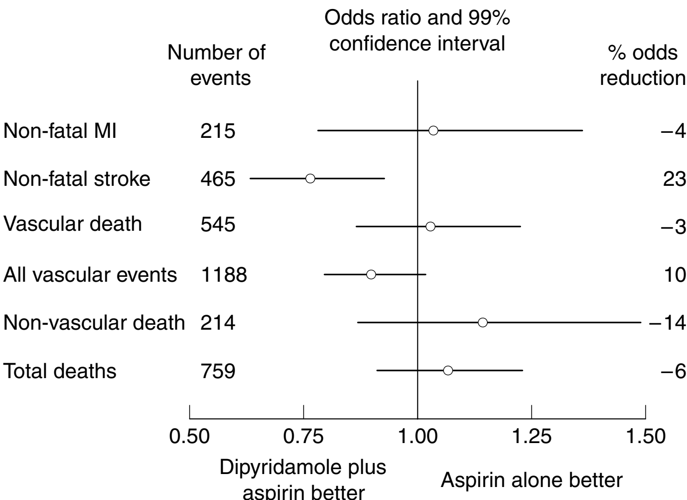


Fig. 70.4. Direct comparisons of the proportional effects of the combination of aspirin and dipyridamole vs. aspirin on vascular events and non-vascular deaths, derived from a total of 8616 patients study in the Antiplatelet Trialists' Collaboration and the second European Stroke Prevention Study[89].

For patients with prior stroke or TIA, four trials involving a total of 2473 patients indicate that the combination of aspirin and dipyridamole is associated with about a 16% (5 to 26%, $P = 0.012$) reduction in the relative risk of serious vascular events[91]. If the risk of stroke and other serious vascular events is about 6.0% per year with aspirin therapy, the combination of aspirin and dipyridamole could reduce it by 16%, to 5.1% per year, which is an ARR of 0.9% per year. The NNT is 111 (100/0.9) compared with aspirin.

The magnitude of this treatment effect continues to be studied in the ongoing European and Australian Stroke Prevention in Reversible Ischaemia (ESPRIT) trial, in which more than 1000 patients (target 4500 patients) with a prior TIA or minor ischemic stroke are being randomized between oral anticoagulation, the combination of aspirin (in any dose between 30 and 325 mg daily) plus dipyridamole (400 mg daily), and aspirin alone[92].

Safety

Headache and gastrointestinal events (e.g. nausea, diarrhea) are the main reasons that patients receiving dipyridamole-containing antiplatelet regimens discontinue treatment prematurely. In the ESPS-2 study, 8.1% of the 1628 patients allocated dipyridamole and 7.1% of the 1631 patients given the combination of aspirin and dipyridamole prematurely ceased their study medication due to headache, compared with 1.9% of the 1631 allocated aspirin and 2.4% if the 1622 given placebo ($P < 0.001$)[82]. Similarly, GI events led to premature discontinuation of study drug in 6.2% of those allocated dipyridamole, and 7.1% of those given the combination of aspirin and dipyridamole compared with 3.7% of each of those allocated aspirin and placebo ($P < 0.001$)[82]. The addition of dipyridamole to aspirin does not appear to exaggerate the excess risk of hemorrhage associated with aspirin.

Cost

The cost of aspirin combined with dipyridamole is Aus$ 350 per patient per year[73]; or about Aus$ 38 850 to treat 111 TIA/stroke patients with aspirin and dipyridamole for 1 year to prevent one more stroke or other serious vascular event than if they were treated with aspirin.

Aspirin and platelet glycoprotein IIb/IIIa receptor antagonists

The combination of aspirin and a parenteral platelet glycoprotein IIb/IIIa blocker given immediately to patients with unstable angina or non-Q-wave MI, or undergoing percutaneous coronary interventions, is significantly more effective than aspirin alone in reducing the 30-day rate of death or non-fatal MI (OR 0.81, 95% CI 0.75 to 0.88)[93]. However, the combination of long-term aspirin and an oral platelet GP IIb/IIIa blocker has, to date, been shown to be no more effective than aspirin alone[94]. The possible reasons for this include an unanticipated thrombotic effect of long-term glycoprotein IIb/IIIa receptor blockade; s excessive drug dose, platelet inhibition and therefore bleeding complication (particularly for patients with creatinine clearance of less than 90 ml/min); inadequate monitoring of platelet inhibition, inadequate duration of treatment and follow-up and the random play of chance (i.e. bad luck). The results from the Blockage of the GP IIb/IIIa Receptor to Avoid Vascular Occlusion (BRAVO) trial are awaited. The primary objective of BRAVO is to compare the incidence of the composite outcome comprising stroke, MI, death from any cause, recurrent ischemia requiring hospitalization, and urgent ischemic-driven revascularizations in patients with TIA, ischemic stroke, acute myocardial infarction, unstable angina, or double bed vascular disease (i.e. peripheral vascular disease combined with cardiovascular or cerebrovascular disease) who are randomized to receive either lotrafiban 30–50 mg b.i.d (depending on age and renal function) or placebo, which is added to their usual prescribed aspirin 75–325 mg daily, for about 2 years. More than 9000 patients will be enrolled from 700 centres in 30 countries thoughout the world.

Cambridge Books Online © Cambridge University Press, 2009

Aspirin and clopidogrel

The combination of aspirin and clopidogrel is the short-term treatment of choice for coronary artery stenting[95,96], and the safety and efficacy of long-term treatment with clopidogrel combined with aspirin is being studied in patients with unstable angina (CURE study) and in high-risk patients with recent TIA and ischemic stroke (MATCH study).

The future of antiplatelet therapy in secondary stroke prevention is likely to be combinations of agents that inhibit platelet adhesion, activation and aggregation at various stages of the process. Ongoing trials are evaluating the relative safety and efficacy of combinations of aspirin with dipyridamole, clopidogrel and oral glycoprotein IIb/III a inhibitors.

Summary of antiplatelet therapy for secondary prevention of stroke and other serious vascular events in patients with TIA and ischemic stroke due to atherothromboembolism

Antiplatelet therapy (aspirin 160–300 mg) should be started as early as possible after an acute TIA or ischemic stroke that is thought to be due to atherothrombembolism.

After the acute phase, aspirin should be continued in a lower dose, 75 mg daily, together with control of vascular risk factors and consideration for revascularization of the symptomatic carotid artery. Adding dipyridamole to aspirin, or substituting clopidogrel for aspirin are marginally but significantly more effective interventions to prevent stroke but they are also substantially more expensive. Clopidogrel is clearly indicated for patients who are intolerant of, or allergic to, aspirin.

The decision to use aspirin, the combination of aspirin and dipyridamole, or clopidogrel in patients with atherothrombotic TIA or ischemic stroke should be determined by the patient's absolute risk of a recurrent stroke or other serious vascular event, the patient's capacity to tolerate the potential adverse effects of the medication, and the patient's (and country's) capacity to pay for the medication. The absolute benefits of antiplatelet regimens are likely to be greater in patients with higher absolute baseline risk of a vascular event because the proportional risk reductions of antiplatelet regimens appear to be similar, irrespective of the baseline absolute risk. For example, among patients with a low baseline absolute risk of subsequent serious vascular events of about 5% per year, antiplatelet therapies reduce this risk by about one-quarter to one-third, which is about 1–2% per year, respectively. In these patients the number needed to treat with antiplatelet therapy to prevent one serious vascular event per year is 50–100 (100/1–2). However, among patients with a higher baseline absolute risk of subsequent serious vascular events of about 40% per year, antiplatelet therapies may reduce this risk by about one-quarter to one-third, which is about 10–13% per year respectively. In these patients the number needed to treat to prevent one serious vascular event per year is only 8–10 (100/10–13).

Antiplatelet therapy in TIA and ischemic stroke patients with atrial fibrillation

In the APT overview, patients with atrial fibrillation experienced similar reductions in vascular events to the other categories of high risk patients[54]. A recent systematic review of antithrombotic therapy for patients with atrial fibrillation showed that, compared with control, aspirin reduced the risk of stroke by 22% (95% CI 2% to 38%). However, adjusted-dose warfarin reduced stroke risk by 62% (95% CI 48% to 72%) compared with control and by 36% (95% CI 14% to 52%) compared with aspirin[97]. Patients with atrial fibrillation and a history of a prior stroke or TIA have a particularly high stroke risk (about 12% per year)[98]. Treatment with warfarin instead of aspirin among 1000 such patients was estimated to prevent 48 strokes per year, with an excess of two major extracranial hemorrhages[97]. Oral anticoagulation (with a target INR of about 2.5) is therefore likely to outweigh the bleeding hazards (and inconvenience of monitoring) for many such patients, providing a net benefit greater than that produced by aspirin. But, aspirin is a suitable alternative for those whose risk of bleeding on anticoagulants is likely to be substantial (for example, those with a history of recent gastrointestinal bleeding, dementia or a tendency to falls).

References

1 MacDonald BK, Cockerell OC, Sander JWAS, Shorvon. SD. The incidence and lifetime prevalence of neurological disorders in a prospective community-based study in the UK. *Brain* 2000; 123: 665–676.
2 Hatano S. Experience from a multicentre stroke register: a preliminary report. *Bull World Health Org* 1976; 54: 541–553.
3 Warlow CP, Dennis MS, van Gijn J et al. *Practical management of stroke* 2000, edition 2; Oxford, UK: Blackwell Scientific Publications.
4 Sudlow CLM, Warlow CP. Comparable studies of the incidence of stroke and its pathological subtypes. Results from an International Collaboration. *Stroke* 1997; 28: 491–499.
5 Hankey GJ. Stroke: how large a public health problem and how the neurologist can help? *Arch Neurol* 1999; 56: 748–754.

Cambridge Books Online © Cambridge University Press, 2009

6 Bonita R, Solomon N, Broad JB. Prevalence of stroke and stroke-related disability: estimates from the Auckland Stroke Studies. *Stroke* 1997; 28: 1898–1902.

7 Bamford J, Sandercock P, Dennis M, Burn J, Warlow C. A prospective study of acute cerebrovascular disease in the community: the Oxfordshire Community Stroke Project – 1981–1986. 2. Incidence, case fatality rates and overall outcome at one year of cerebral infarction, primary intracerebral and subarachnoid hemorrhage. *J Neurol Neurosurg Psychiat* 1990; 53: 16–22.

8 Murray CJL, Lopez AD. Mortality by cause for eight regions of the world. Global Burden of Disease Study. *Lancet* 1997; 349: 1269–1276.

9 Wade DT. Functional abilities after stroke: measurement, natural history and prognosis. *J Neurol Neurosurg Psychiat* 1987; 50: 177–182.

10 Murray CJL, Lopez AD. Global mortality, disability and the contribution of risk factors. Global Burden of Disease Study. *Lancet* 1997; 349: 1436–1442.

11 Hankey GJ, Jamrozik K, Broadhurst RJ et al. Long-term risk of first recurrent stroke in the Perth Community Stroke Study. *Stroke* 1998; 29: 2491–2500.

12 Raichle M. The pathophysiology of brain ischaemia. *Ann Neurol* 1983; 13: 2–10.

13 Hunter J. A treatise on the blood, inflammation and gunshot wounds. In Palmer JF, ed. *The work of John Hunter* 1937; London: Longman.

14 Kullo IJ, Edwards WD, Schwartz RS. Vulnerable plaque: pathobiology and clinical implications. *Ann Intern Med* 1998; 129: 1050–1060.

15 Ross R. Atherosclerosis – an inflammatory disease. *N Engl J Med* 1999; 340: 115–126.

16 Davies MJ. The composition of coronary artery plaques. *N Engl J Med* 1997; 336: 1312–1314.

17 Davies MJ. Stability and instability: two faces of coronary atherosclerosis. *Circulation* 1996; 94: 2013–2020.

18 Burke AP, Farb A, Malcolm GT, Liang Y-H, Smialek J, Virmani R. Coronary risk factors and plaque morphology in men with coronary disease who died suddenly. *N Engl J Med* 1997; 336: 1276–1282.

19 Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993; 362: 801–809.

20 Fuster V, Fallon JT, Badimon JJ, Nemerson Y. The unstable atherosclerotic plaque: clinical significance and therapeutic intervention. *Thromb Haemost* 1997; 78(1): 247–255.

21 DeGraba TJ. Expression of inflammatory mediators and adhesion molecules in human atherosclerotic plaque. *Neurology* 1997; 49(Suppl 4): S15–S19.

22 Libby P. Molecular bases of the acute coronary syndromes. *Circulation* 1995; 91: 2844–2850.

23 Hunt BJ, Jurd KM. Endothelial cell activation. *Br Med J* 1998; 316: 1328–1329.

24 Cheng GC, Loree HM, Kamm RD, Fishbein MC, Lee RT. Distribution of circumferential stress in ruptured and stable atherosclerotic lesions: a structural analysis with histopathological correlation. *Circulation* 1993; 87: 1179–1187.

25 Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med* 1997; 336: 973–979.

26 Danesh J, Whincup P, Walker M et al. Low grade inflammation and coronary heart disease: prospective study and updated meta-analysis. *Br Med J* 2000; 321: 199–204.

27 Danesh J, Collins R, Peto R. Chronic infections and coronary heart disease: is there a link? *Lancet* 1997; 350: 430–436.

28 Gurfinkel E, Bozovich G, Daroco A, Beck E, Mautner B, for the ROXIS Study Group. Randomised trial of roxithromycin in non-Q-wave coronary syndromes: ROXIS Pilot Study. *Lancet* 1997; 350: 404–407.

29 Bittner V. Atherosclerosis and the immune system. *Arch Intern Med* 1998; 158: 1395–1396.

30 Wald NJ, Law MR, Morris JK, Zhou X, Wong Y, Ward ME. *Chlamydia pneumoniae* infection and mortality from ischemic heart disease: large prospective study. *Br Med J* 2000; 321: 204–207.

31 Danesh J, Whincup P, Walker M et al. *Chlamydia pneumoniae* IgG titres and coronary heart disease: prospective study and meta-analysis. *Br Med J* 2000; 321: 208–213.

32 Koenig W. Heart disease and the inflammatory response. *Br Med J* 2000; 321: 187–188.

33 Grau AJ, Buggle F, Ziegler C et al. Association between acute cerebrovascular ischaemia and chronic and recurrent infection. *Stroke* 1997; 28: 1724–1729.

34 Bova IY, Bornstein NM, Korczyn AD. Acute infection as a risk factor for ischemic stroke. *Stroke* 1996; 27: 2204–2206.

35 Wimmer ML, Sandmann-Strupp R, Saikku P, Haberl RL. Association of chlamydial infection with cerebrovascular disease. *Stroke* 1996; 27: 2207–2210.

36 Markus HS, Mendall MA. *Helicobacter pylori* infection: a risk factor for ischemic cerebrovascular disease and carotid atheroma. *J Neurol Neurosurg Psychiat* 1998; 64: 104–107.

37 Yamashita K, Ouchi K, Shirai M, Gondo T, Makazawa T, Ito H. Distribution of *Chlamydia pneumoniae* infection in the atherosclerotic carotid artery. *Stroke* 1998; 29: 773–778.

38 Cook PJ, Honeybourne D, Lip GYH, Beevers G, Wise R, Davies P. *Chlamydia pneumoniae* antibody titers are significantly associated with acute stroke and transient cerebral ischemia. The West Birmingham Stroke Project. *Stroke* 1998; 29: 404–410.

39 Grau AJ, Buggle F, Becher H et al. Recent bacterial and viral infection is a risk factor for cerebreovascular ischemia: clinical and biochemical studies. *Neurology* 1998; 50: 196–203.

40 Rothwell PM, Villagra R, Gibson R, Donders RCJM, Warlow CP. Evidence of a chronic systemic cause of instability of atherosclerotic plaques. *Lancet* 2000, 355: 19–24.

41 George JN. Platelets. *Lancet* 2000; 355: 1531–1539.

42 Hynnes RO. Integrins: versatility, modulation and signalling in cell adhesion. *Cell* 1992; 69: 11–25.

Cambridge Books Online © Cambridge University Press, 2009

43 Hirsh J, Weitz JI. New antithrombotic agents. *Lancet* 1999; 353: 1431–1436.
44 Coller BS. Platelet GPIIb/IIIa antagonists: the first anti-integrin receptor therapeutics. *J Clin Invest* 1997; 99: 1467–1471.
45 Topol EJ, Byzova TV, Plow EF. Platelet GPIIb–IIIa blockers. *Lancet* 1999; 353: 223–227.
46 Schror K, Braun M. Platelets as a source of vasoactive mediators. *Stroke* 1990; 21 (Suppl. IV): IV32–35.
47 Patrono C, Ciabattoni G, Davi G. Thromboxane biosynthesis in cardiovascular diseases. *Stroke* 1990; 21 (Suppl. IV): IV130–133.
48 D'Andrea G, Cananzi AR, Perini F. Platelet function in acute ischemic stroke: relevance of granule secretion. *Cerebrovasc Dis* 1994; 4: 163–169.
49 van Kooten F, Ciabattoni G, Patrono C, Dippel DW, Koudstaal PJ. Platelet activation and lipid peroxidation in patients with acute ischemic stroke. *Stroke* 1997; 28: 1557–1563.
50 Furie B, Furie BC. Molecular and cellular biology of blood coagulation. *N Engl J Med* 1992; 326: 800–806.
51 Broze GJ Jnr. The role of tissue factor pathway inhibitor in a revised coagulation cascade. *Semin Haematol* 1992; 29(3): 159–169.
52 Rosenberg RD, Rosenberg JS. Natural anticoagulant mechanisms. *J Clin Invest* 1984; 74(1): 1–6.
53 Gubitz G, Sandercock P, Counsell C. Antiplatelet therapy for acute ischemic stroke (Cochrane Review). In *The Cochrane Library*, Issue 3, 2000. Oxford: Update Software.
54 Antiplatelet Trialists' Collaboration. Collaborative overview of randomised trials of anti platelet therapy – I: Prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. *Br Med J* 1994; 308: 81–106.
55 Multicentre Acute Stroke Trial-Italy (MAST-I) Group. Randomised controlled trial of streptokinase, aspirin, and combination of both in treatment of acute ischemic stroke. *Lancet* 1995; 346: 1509–1514.
56 International Stroke Trial Collaborative Group. The International Stroke Trial (IST): a randomised trial of aspirin, subcutaneous heparin, both, or neither among 19435 patients with acute ischemic stroke. *Lancet* 1997; 349: 1569–1581.
57 CAST (Chinese Acute Stroke Trial) Collaborative Group. CAST: randomised placebo- controlled trial of early aspirin use in 20000 patients with acute ischemic stroke. *Lancet* 1997; 349: 1641–1649.
58 Berge E, Abdelnoor M, Nakstad PH, Sandset PM, on behalf of the HAEST Study Group. Low molecular-weight heparin versus aspirin in patients with acute ischemic stroke and atrial fibrillation: a double-blind randomised study. *Lancet* 2000; 355: 1205–1210.
59 Ciufetti G, Aisa G, Mercuri M, Lombardini R, Paltriccia R, Neri C, Senin U. Effects of ticlopidine on the neurologic outcome and the hemorheologic pattern in the postacute phase of ischemic stroke: a pilot study. *Angiology* 1990; 41: 505–511.
60 Ohtomo E, Kutsuzawa T, Kogure K et al. Clinical usefulness of OKY-046 on the acute stage of cerebral thrombosis – double blind trial in comparison with placebo. *Rinsho Iyaki* (*J Clin Therap Med*) 1991; 7(2): 353–388.
61 The Abciximab in Ischemic Stroke Investigators. Abciximab in acute ischemic stroke. A randomised, double-blind, placebo-controlled, dose-escalation study. *Stroke* 2000; 31: 601–609.
62 Chen Z, Sandercock P, Pan H et al. Indications for early aspirin use in acute ischemic stroke. A combined analysis of 40,000 randomized patients from the Chinese Acute Stroke Trial and the International Stroke Trial. *Stroke* 2000; 31: 1240–1249.
63 Antithrombotic Trialists' Collaboration. Prevention of death, myocardial infarction, and stroke by antiplatelet therapy in high risk patients. *Br Med J* (in press), 2002.
64 Algra A, van Gijn J. Cumulative meta-analysis of aspirin efficacy after cerebral ischaemia of arterial origin. *J Neurol Neurosurg Psychiat* 1999; 66: 255.
65 Patrono C. Aspirin as an antiplatelet drug. *N Engl J Med* 1994; 330: 1287–1293.
66 UK- TIA Study Group. The United Kingdom transient ischemic attack (UK-TIA) aspirin trial: final results. *J Neurol Neurosurg Psychiat* 1991; 54: 1044–1054.
67 Taylor DW, Barnett HJM, Haynes RB et al. Low-dose and high-dose acetylsalicylic acid for patients undergoing carotid endarterectomy: a randomised controlled trial. *Lancet* 1999; 353: 2179–2184.
68 Dutch TIA Trial Study Group. A comparison of two doses of aspirin (30 mg vs 283 mg a day) in patients after a transient ischemic attack or minor ischemic stroke. *N Engl J Med* 1991; 325: 1261–1266.
69 Roderick PJ, Wilkes HC, Meade TW. The gastrointestinal toxicity of aspirin: an overview of randomised controlled trials. *Br J Clin Pharmacol* 1993; 35: 241–249.
70 Weil J, Colin-Jones D, Langman M et al. Prophylactic aspirin and the risk of peptic ulcer bleeding. *Br Med J* 1995; 310: 827–830.
71 Kelly JP, Kaufman DW, Jurgelon JM, Sheehan J, Koff RS, Shapiro S. Risk of aspirin-associated major upper-gastrointestinal bleeding with enteric-coated or buffered product. *Lancet* 1996; 348: 1413–1416.
72 He J, Whelton PK, Vu B, Klag MJ. Aspirin and risk of hemorrhagic stroke. A meta-analysis of randomised controlled trials. *J Am Med Assn* 1998; 280: 1930–1935.
73 Hankey GJ, Warlow CP. Treatment and secondary prevention of stroke: evidence, costs, and effects on individuals and populations. *Lancet* 1999; 354: 1457–1463.
74 Dyken ML, Barnett HJM, Easton D et al. Low-dose aspirin and stroke. 'It ain't necessarily so'. *Stroke* 1992; 23: 1395–1399.
75 Barnett HJM, Kaste M, Meldrum H, Eliasziw M. Aspirin dose in stroke prevention. Beautiful hypotheses slain by ugly facts. *Stroke* 1996; 27: 588–592.
76 Patrono C, Roth G. Aspirin in ischemic cerebrovascular disease. How strong is the case for a different dosing regimen? *Stroke* 1996; 27: 756–760.
77 van Gijn J. Low doses of aspirin in stroke prevention. *Lancet* 1999; 353: 2172–2173.

Cambridge Books Online © Cambridge University Press, 2009

78 Patrono C, Ciabattoni G, Patrignani P et al. Clinical pharmacology of platelet cyclooxygenase inhibition. *Circulation* 1985; 72: 1177–1184.

79 Johnson ES, Lanes SF, Wentworth CE 3d et al. A metaregression analysis of the dose-response effect of aspirin on stroke. *Arch Intern Med* 1999; 159: 1248–1253.

80 SALT Collaborative Group. Swedish aspirin low-dose trial (SAL T) of 75mg aspirin as secondary prophylaxis after cerebrovascular ischemic events. *Lancet* 1991; 338: 1345–1349.

81 Juul-Möller S, Edvardsson N, Jahnmatz B, Rosen A, Sorensen S, Ömblus R et al. Double- blind trial of aspirin in primary prevention of myocardial infarction in patients with stable chronic angina pectoris. *Lancet* 1992; 340: 1421–1425.

82 Diener HC, Cunha L, Forbes C, Sivenius J, Smets P, Lowenthal A. European stroke prevention study 2. Dipyridamole and acetylsalicylic acid in the secondary prevention of stroke. *J Neurol Sci* 1996; 143: 1–13.

83 Gent M, Beaumont D, Blanchard J et al. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischemic events (CAPRIE). *Lancet* 1996; 348: 1329–1338.

84 Hankey GJ, Sudlow CLM, Dunbabin DW. Thienopyridines or aspirin to prevent stroke and other serious vascular events in patients at high risk of vascular disease. *Stroke* 2000; 31: 1779–1784.

85 Steinhubl SR, Tan WA, Foody JM, Topol EJ, for the EPISTENT investigators. Incidence and clinical course of thrombotic thrombocytopenic purpura due to ticlopidine following coronary stenting. *J Am Med Assn* 1999; 281: 806–810.

86 Torok TJ, Holman RC, Chorba TL. Increasing mortality from thrombotic thrombocytopenic purpura in the United States: An analysis of national mortality data, 1968–1991. *Am J Hematol* 1995; 50: 84–90.

87 Bennett CL, Connors JM, Carwile JM et al. Thrombotic thrombocytopenic purpura associated with clopidogrel. *N Engl J Med* 2000; 342: 1773–1777.

88 Bennett CL, Connors JM, Moake JL. Clopidogrel and thrombotic thrombocytopenic purpura. *N Engl J Med* 2000; 343: 1191–1194.

89 Hankey GJ. Clopidogrel and thrombotic thrombocytopenic purpura. *Lancet* 2000; 356: 269–270.

90 Wilterdink JL, Easton JD. Dipyridamole plus aspirin in cerebrovascular disease. *Arch Neurol* 1999; 56: 1087–1092.

91 Algra A, van Gijn J, Koudstaal PJ. Secondary prevention after cerebral ischaemia of presumed arterial origin: is aspirin still the touchstone? *J Neurol Neurosurg Psychiat* 1999; 66: 557–559.

92 De Schryver ELLM, on behalf of the European/Australian Stroke Prevention in Reversible Ischaemia Trial (ESPRIT) Group. Design of ESPRIT: an international randomised trial for secondary prevention after non-disabling cerebral ischaemia of arterial origin. *Cerebrovasc Dis* 2000; 10: 147–150.

93 Topol EJ. Toward a new frontier in myocardial reperfusion therapy. Emerging platelet preeminence. *Circulation* 1998; 97: 211–218.

94 Heeschen C, Hamm CW. Difficulties with oral platelet glycoprotein IIb/IIIa receptor antagonists. *Lancet* 2000; 355: 330–331.

95 Bertrand ME, Rupprecht H-J, Urban P, Gerschlick AH. Comparative safety of ticlopidine and clopidogrel in coronary stent patients: data from CLASSICS (Abstract). *Circulation* 1999; 100 (suppl I): I-620.

96 Moussa I, Oetgen M, Roubin G et al. Effectiveness of clopidogrel and aspirin versus ticlopidine and aspirin in preventing stent thrombosis after coronary stent implantation. *Circulation* 1999; 99: 2364–2366.

97 Hart RG, Benavente O, McBride R, Pearce LA. Antithrombotic therapy to prevent stroke in patients with atrial fibrillation: a meta-analysis. *Ann Intern Med* 1999; 131: 492–501.

98 European Atrial Fibrillation Trial Study Group. Secondary prevention in non-rheumatic atrial fibrillation after transient ischemic attack or minor stroke. *Lancet* 1993; 342: 1255–1262.

Cambridge Books Online © Cambridge University Press, 2009

71

Antiplatelet treatment in peripheral vascular disease

Raymond Verhaeghe

Department of Molecular and Cardiovascular Research, University of Leuven, Belgium

Progressive atherosclerosis complicated by thromboembolic events is by far the most common cause of peripheral arterial disease. Atherothrombosis, the fashionable term to describe the stenotic/thrombotic process, is a generalized disorder, which is almost invariably ubiquitous and therefore any patient who presents with symptoms or signs suggestive of arterial disease in one vascular bed is likely to have other territories involved as well. The common risk factors for atherosclerosis also apply to peripheral arterial disease, but the order of importance varies: smoking correlates most strongly with disease of the leg arteries and predicts its progression. The disease in the leg arteries remains clinically silent as long as no hemodynamically significant obstructive lesions are formed. Subclinical disease is detected with the aid of simple non-invasive tests in three to four times as many middle-aged adults as is symptomatic disease. Intermittent claudication, leg pain on exercise that is relieved by rest, develops when a drop in driving pressure occurs. Progression of the disease to the extent that the blood flow becomes insufficient to meet the metabolic and nutritional demands of resting tissues is clinically manifested by rest pain and skin lesions at the foot. A Working Group defined chronic critical leg ischemia as the presence of restpain requiring regular analgesia for more than 2 weeks and of an ankle systolic pressure of less than 50 mm Hg (and/or toe systolic pressure of less than 30 mm Hg) with or without skin ulceration or gangrene of toes or foot[1].

The pathophysiology of atherothrombosis and particularly the contribution of platelets is detailed in Chapter 30. The importance attributed to platelets led to development and application of many tests which aim to detect ‘hyperactive’ platelets with the hope that they would predict future thrombotic events. Studies which assess platelet specific proteins or which test platelet adhesion or aggregation in vitro have produced conflicting data in patients with peripheral arterial disease; they may be too insensitive to detect in vivo platelet activation. Measurement of urinary excretion of 11-dehydro-thromboxane B_2 appeared to provide a more sensitive index of platelet activation in a study on 64 claudication patients. The rate of excretion of the thromboxane metabolite was related to the presence of cardiovascular risk factors and predicted future thrombotic complications of peripheral arterial disease[2,3]. Decreased availability of biologically active NO in the blood vessel wall, in accordance with the clinical severity of the disease, is one possible mechanism of platelet activation in these patients[4].

The fate of the patient with intermittent claudication is relatively benign as regards local arterial disease in the legs, but is increasingly malignant in terms of serious fatal and non-fatal cardiovascular events[5]. A fair minority of claudicants require surgical or endovascular intervention over 5 years after diagnosis because of increasing severity of their functional handicap or because of deterioration to critical ischemia. Only 2% will ever need a major amputation. By contrast, they have a high prevalence of coronary artery and cerebrovascular disease and therefore a significantly increased risk of stroke, myocardial infarction and cardiovascular death. Their 5-, 10-, and 15-year mortality rates from all causes are approximately 30, 50 and 70%, respectively, 2.5 times higher than expected. Thus the real danger is not losing a leg but rather suffering premature cardiovascular complications or death (Fig. 71.1).

Patients with critical limb ischemia carry a worse prognosis, not only in terms of mortality and cardiovascular morbidity, but also as regards the fate of their leg. Those who are either unsuitable for revascularization or in whom revascularization failed are probably the subgroup of patients with leg arterial disease with the worst outcome: placebo groups of pharmacotherapy trials that select such patients indicate a 20% death rate and a 40% major amputation rate at 6 months.

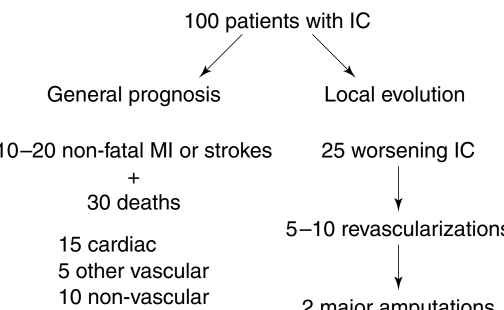


Fig. 71.1. Natural history of patients with intermittent claudication over 5 years after diagnosis. IC: intermittent claudication; MI: myocardial infarction.

Management of patients with arterial disease in the leg involves several aspects, of which risk factor control is of primary importance. This chapter focuses on the use of antiplatelet drugs which are being prescribed with the aim of improving the general prognosis and retarding the local progression of atherosclerosis in the legs. Although recent studies have reported similar findings with other classes of pharmacological agents such as statins and angiotensin-converting-enzyme inhibitors, these drugs are not discussed here.

Do antiplatelet drugs prevent vascular events?

In general, patients with symptomatic peripheral arterial disease receive aspirin, ticlopidine or clopidogrel to decrease the incidence of thrombo-embolic vascular events. This aim is not well demonstrated in this category of patients. Particularly, the data on aspirin are poor: no single study with clinical endpoints yields significant results. The collaborative overview of randomized trials of antiplatelet therapy, the meta-analysis of the Antiplatelet Trialists' Collaboration, calculated that the vascular risk was reduced to a similar extent (odds reduction of 27 ± 2%) in a category of high-risk patients of mixed origin as in patients with prior myocardial infarction (25 ± 4%), with acute myocardial infarction (29 ± 4%) or with prior stroke/TIA (22 ± 4%). This mixed category was subdivided, and peripheral vascular disease was one of the major subgroups. In this subgroup, the risk reduction in patients with intermittent claudication (almost 3300 patients in 22 trials) was not significant. However, there was no evidence of significant heterogeneity either between the results of the subgroups. In addition, in almost all of the subgroups results appeared to favour active treatment. The authors therefore concluded that it 'may well be that antiplatelet therapy is likely to be protective for any high-risk patients with clinically evident occlusive vascular disease unless there is some special contra-indication'[6]. The popular name of this meta-analysis is 'the Aspirin Papers', but scrutiny of the analysed trials indicates that the number of included patients on ticlopidine by far exceeds that on aspirin. Another ticlopidine trial was published later: it enrolled 615 patients of whom 304 were assigned to ticlopidine treatment and 311 to placebo for 24 weeks. Four patients in the ticlopidine group and 12 in the placebo group experienced a vascular event (sudden death, myocardial infarction or stroke) during the follow-up period (relative risk 2.93 with 95% CI 0.96–8.99; risk reduction 68%; $P = 0.04$; intention-to-treat analysis). The risk reduction was consistent when subgroups (diabetic and non-diabetic patients) were analysed separately[7]. Clopidogrel (75 mg daily), the new thienopyridine, was compared to aspirin (325 mg daily) in the large CAPRIE-trial which studied patients with stroke, myocardial infarction or peripheral arterial disease. The subgroup of 6452 patients with symptomatic peripheral arterial disease followed for 1–3 years sustained significantly fewer endpoints (stroke, myocardial infarction and vascular death) when treated with clopidogrel than with aspirin: the yearly event rate was 3.71 and 4.86% in the two treatment groups, respectively (relative risk reduction of 24%, $P = 0.0028$). Virtually all of the benefit obtained with clopidogrel in this study, was observed in this subgroup of patients[8]. A recent meta-analysis evaluated the evidence of the effectiveness of individual antithrombotic drugs to reduce vascular events and/or mortality in patients with intermittent claudication and concluded that only ticlopidine and clopidogrel have proven efficacy in level 1 studies (randomized and double-blind or assessor-blind studies)[9].

Although ticlopidine and clopidogrel appear to have better credentials than aspirin, most authorities still advise to use aspirin as a first choice and to change to ticlopidine, or preferentially to clopidigrel because of its better safety profile, in patients who do not tolerate aspirin or who experience a major cardiovascular event despite aspirin intake. The preference for aspirin cannot be based on direct evidence, but on analogy with coronary and cerebral disease where efficacy is well documented. Cost–benefit is an important aspect to justify this choice, and this applies as well for clopidogrel as for ticlopidine[10,11].

Should antiplatelet drugs be started before claudication symptoms develop? In patients with subclinical disease (thus with a decreased ankle–arm pressure index) this would seem reasonable since they appear to have the same increased risk of cardiovascular events and death found in

Cambridge Books Online © Cambridge University Press, 2009

Table 71.1. Progression of atherosclerosis in the leg

Aspirin (-dipyridamole)	
Schoop et al.[12]: femoral artery occlusion rate ($n=300$ – follow-up: 4.5 years)	
	%
Placebo	58
Aspirin (330 mg t.i.d.)	20[a]
Aspirin–dipyridamole (330 mg–75 mg t.i.d.)	34[a]
Hess et al.[13]: angiographic progression ($n=199$ – follow-up: 2 years)	
	Score
Placebo	+6.2
Aspirin (330 mg t.i.d.)	+4.4
Aspirin–dipyridamole (330 mg–75 mg t.i.d.)	+2.2[a]
Ticlopidine	
Stiegler et al.[14] angiographic progression ($n=43$ – follow-up: 1 year)	
	Score
Placebo	+4.2
Ticlopidine (250 mg b.i.d.)	+0.1[a]

Note:
[a] significantly different from placebo.

claudicants. However, a formal study to sustain this view has not been published.

Antiplatelet treatment and ischemic symptoms in the limb

The ultimate 'local' goal of therapy in chronic arterial occlusive disease is to relieve ischemic symptoms (intermittent claudication and rest pain) and to prevent progression of atherosclerosis that might lead to tissue and limb loss. Three trials in the 1980s studied the angiographic evolution as a surrogate endpoint: in one antiplatelet treatment reduced the rate of progression to total occlusion and in the other two treatment reduced an angiographic score which evaluates the extent and severity of the obstructive lesions[12–14](Table 71.1). One of the two latter reports mentions newly developed occlusions in arterial segments as well: 30/1838 with active treatment (aspirin or aspirin/dipyridamole) vs. 34/972 with placebo (odds ratio 0.46, 95% CI 0.27–0.77)[13]. The clinical relevance of the reported changes is not easily appreciated.

A few studies focused on the need for intervention as a clinical endpoint. The US Physicians' Health Study reports

Table 71.2. Need for further vascular surgery in ticlopidine trials

Authors[ref]	Number of patients	Placebo	Ticlopidine
Bergqvist et al.[16]	total	232	236
	surgery	23	16
Blanchard et al.[7]	total	311	304
	surgery	8	1

that aspirin (325 mg taken on alternate days) significantly reduces the need for peripheral arterial surgery in males without a history of myocardial infarction, stroke or transient cerebral ischemia. During an average 5 year follow-up period (over 100 000 person years) 56 trial participants underwent arterial surgery: 20 assigned to aspirin and 36 to placebo (relative risk with aspirin 0.54, 95% CI 0.30–0.95; $P=0.03$). The benefit was more pronounced in the subset of individuals who reported claudication at entry into the study. By contrast, the incidence of self-reported new claudication was almost identical in the two groups (112 and 109, respectively). Thus, aspirin appeared to have more impact on the later than on the early stages of the disease, suggesting an antithrombotic rather than an anti-atherogenic effect[15]. The number of patients with further vascular surgery was also assessed in two studies with ticlopidine (Table 71.2)[7,16]: a statistically significant reduction in need for revascularization was observed when the two trials were analyzed together (odds ratio 0.62, 95% CI 0.41–0.93)[9].

Walking distance provides information on the functional capacity in patients with impaired arterial circulation in the leg. It is a soft, but clinically relevant, endpoint for patients with intermittent claudication. Several studies with ticlopidine provide data on the evolution of walking distance. A meta-analysis on four placebo-controlled trials[17,18] and a fifth controlled study[19] all conclude to a significantly better evolution of walking capacity with ticlopidine. By contrast, ticlopidine did not improve walking distance significantly over placebo in the Argentinian multicenter study[7].

Statistically significant improvement of walking distance was obtained with several other drugs which exhibit antiplatelet effects, although their use in leg arterial disease does not intend to compete with aspirin or the thienopyridines. The benefit with pentoxifylline has not been very consistent throughout the many conducted studies[20]. The drug improves red cell deformability, lowers fibrinogen levels and decreases platelet reactivity; its clinical effects may thus stem from other pharmacological properties than

Cambridge Books Online © Cambridge University Press, 2009

from its weak antithrombotic action. Cilostazol acts by selectively inhibiting phosphodiesterase type 3, an enzyme that breaks down cAMP. The final result is a lower level of intracellular Ca^{2+} within platelets which, in turn, represses platelet activity. In addition to its antiplatelet effects, cilostazol acts as an arterial vasodilator: it inhibits the function of contractile proteins by an analogous mechanism. Synthesis of prostaglandins is not affected. The drug was first marketed in Japan for patients with intermittent claudication and ischemic leg ulcers. Recently, 6 multicentre placebo-controlled trials were conducted in the US. They involved more than 2000 patients with intermittent claudication and established the efficacy of cilostazol to improve walking distance in these patients[21,22]. Prostaglandins with antiplatelet and vasodilatory effects (mainly E_1 and I_2) and some of their analogues have been used primarily in studies on patients with critical limb ischemia; the reported results varied but there is some evidence that the prostacyclin analogue iloprost may improve amputation-free survival in these patients. A prodrug of prostaglandin E_1 administered intravenously appeared to have a positive effect on walking distance of claudication patients in two placebo-controlled studies of short duration[23,24]. Beraprost is an orally active prostaglandin I_2 analogue that was recently tested in a study on 549 patients with intermittent claudication. Their walking distance improved significantly over 6 months in comparison with placebo. In addition, the incidence of cardiovascular events was markedly reduced (mainly arterial thrombosis of the leg) but the trial was not powered to study this endpoint[25]. Effects of other less common antiplatelet agents are discussed in Chapter 65.

No published study has yet proved that aspirin will prevent the primary claudication symptoms in a healthy population. Because such a study would require very large numbers to be enrolled, an alternative approach is to select a population at higher risk, such as individuals with a (still) asymptomatic decrease in ankle pressure index or patients with diabetes. Such studies are under way.

Is antiplatelet treatment useful in arterial surgery?

Thrombosis, restenosis at the anastomosic sites and late occlusion are the main problems after implantation of a graft or after a thrombendarterectomy. Antithrombotic drugs are primarily expected to decrease the risk of early thrombosis; in a clinical setting, they have never been shown to influence intimal hyperplasia, the mechanism underlying restenosis.

To what extent does antiplatelet therapy contribute to maintain patency of peripheral arterial reconstructions? Clinical trials in this field are not numerous, usually small, and fewer than half of them have a randomized and double-blind design. Many are single centre studies including a mixture of different surgical procedures and patients with varying degrees of lower limb ischemia[26]. The American College of Chest Physicians (ACCP) Consensus Conference (2001) recommends not to use antithrombotic drugs to maintain patency of vascular reconstructions involving high-flow low-resistance arteries greater than 6 mm in diameter. Thus aorto-iliac and aorto-femoral grafts do not require antithrombotic prophylaxis because thrombotic occlusion is unusual and patency rates in the range of 80 to 90% can be expected after 5 to 10 years[27]. An exception to this rule are long axillo-femoral bypasses that appear particularly vulnerable to thrombotic occlusion.

By contrast, reconstructions below the inguinal ligament are more prone to thrombosis because of the lower flow rates, the length of the bypasses and their frequent bending at flexion points of knee and groin. In addition, prosthetic grafts should be distinguished from vein grafts because they have an inherently higher thrombogenicity. As a consequence, the ACCP Consensus Conference proposes the use of aspirin (with or without dipyridamole) in patients having prosthetic infrainguinal bypass operations and to start antithrombotic therapy preoperatively. This recommendation is largely based on six trials published between 1982 and 1997[28–33]. They are summarized in Table 71.3. A recent meta-analysis included five randomized controlled trials comparing long-term aspirin (with or without other antiplatelet therapy) with placebo after infrainguinal bypass surgery[26]. In 423 patients treated with antiplatelet drugs, 120 (28.4%) of the bypasses occluded compared with 144 (36.6%) occlusions in 393 randomized placebo-treated patients, giving a relative risk of 0.78 (95% CI 0.64–0.95) or a proportional risk reduction of 22%; the absolute risk reduction was 8.2%. No distinction was made between prosthetic and venous bypasses. The importance of graft material is emphasized in another meta-analysis on 11 randomized controlled trials of platelet inhibitors after peripheral bypass procedures involving 2302 patients. In this meta-analysis there was a clear treatment benefit of platelet inhibitors but also a significant heterogeneity which was explained by the proportion of patients with a prosthetic graft in an individual trial. Aspirin did not appear to prevent occlusion of vein grafts[34]. The Antiplatelet Trialists' Collaboration meta-analysis[35] on 11 randomized trials (a large number are the same trials), also included over 2000 patients with peripheral vascular grafts and concluded that antiplatelet drugs reduce the inci-

Cambridge Books Online © Cambridge University Press, 2009

Table 71.3. Antiplatelet therapy in infrainguinal grafts

Authors[ref]	Patients	Type of graft (*n*)	Follow-up	Patency (per cent)	Comment
Green et al.[28]	49	Prost	1 year	Asp: 88 Asp-dip: 62.5 Placebo: 25	
Goldman, McCollum[29]	53	Prost	1 year	Asp-dip: 67 Placebo: 36	$P<0.05$
Kohler et al.[30]	100	Vein 51 Prost 51	2 years life table	Asp-dip: 67 Placebo: 57	Not significant treatment started postsurgery
Clyne et al.[31]	111	Vein 93 Prost 55	1 year life table	Asp-dip: 84 Placebo: 65	$P=0.012$ Benefit entirely in prosthetic grafts within first month
McCollum et al.[32]	549	Vein	3 years	Asp-dip: 61 Placebo: 60	More MI and stroke with placebo
Becquemin et al.[33]	243	Vein	2 years	Ticlopidine: 82 Placebo: 63	$P=0.002$

Note:
Prost: prosthetic; Asp: aspirin; Dip: dipyridamole.

dence of graft occlusion at the end of the (variable) follow-up period by one-third (from 24 to 16%). This conclusion is, to some extent, upheld in a new meta-analysis that included trials with several drugs and several types of revascularization procedures: mainly aspirin associated with dipyridamole and ticlopidine appeared to improve the outcome[36]. The multitude of meta-analyses to answer the same question – do antiplatelet drugs improve the patency rate of vascular grafts – illustrates the difficulty and uncertainty in the field due to the lack of large studies with homogenous groups.

Up to now, the French multicentre study[33] is the only controlled trial in which an antiplatelet drug reduces the rate of late occlusion in an homogenous population of saphenous vein bypass grafts below the knee. Therefore, there is currently no strict recommendation for the use of antiplatelet drugs to maintain patency of saphenous vein bypass grafts. Some surgeons even favour the use of oral anticoagulants in patients with this type of vascular graft. A small study suggested adding oral anticoagulants to aspirin to improve patency of infrainguinal vein grafts at high risk of thrombosis[37]. The recent Dutch Bypass Oral anticoagulants or aspirin study (BOA) investigated whether either of these treatments prevented more effectively than the other bypass complications after infrainguinal bypass surgery in 2690 patients, but found no significant difference in overall occlusion rate. The frequency of occlusion was about 14% per 100 patient–years in each treatment group. However, analysis stratified for graft material showed a lower risk of autologous vein–graft occlusion in the oral anticoagulation group than in the aspirin group; conversely, aspirin appeared more effective in patients with a non-venous graft (Fig. 71.2)[38]. Despite careful control of oral anticoagulant therapy (there is a well-organized system of anticoagulation clinics in the Netherlands), bleeding complications were twice as common with oral anticoagulation as with antiplatelet therapy. There were 18 cases of intracranial bleeding, 8 of which were fatal, in the oral anticoagulation group, vs. 4 (3 fatal) in the aspirin group. This surplus was offset by a lower incidence of non-hemorrhagic stroke: 21 with oral anticoagulants vs. 45 with aspirin.

Surgical patients may benefit from antiplatelet therapy to reduce long-term cardiovascular mortality and morbidity as well as non-operated patients.

Antiplatelet drugs in percutaneous endovascular revascularization

The 1998 recommendations of the ACCP Consensus Conference for patients undergoing percutaneous transluminal angioplasty (PTA) are summarized in Table 71.4[10]. A European expert group suggested replacing ticlopidine

Cambridge Books Online © Cambridge University Press, 2009

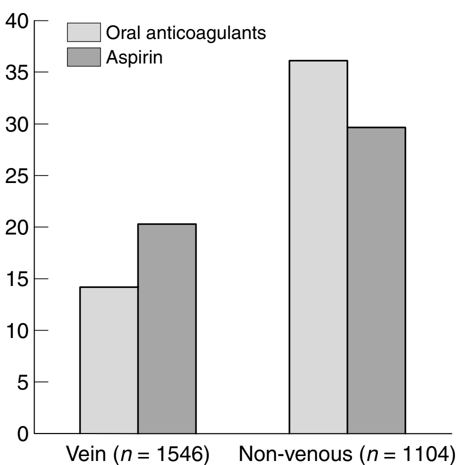


Fig. 71.2. Incidence of graft occlusion with aspirin and oral anticoagulants in the Dutch Bypass Oral anticoagulants or aspirin study[36].

with clopidogrel which has a similar clinical efficacy but a better safety profile[11]. The proposal largely relies on extrapolation from data in coronary angioplasty. The 2001 ACCP document keeps silence on the issue[27].

Aspirin (earlier frequently combined with dipyridamole) started the day before the procedure is the usual antithrombotic drug in most centres. Historically, this practice appears to be based on a few rather small and methodologically questionable studies of short duration from the pioneering period. Studies with longer follow-up after PTA rarely distinguished early reocclusion from late restenosis and/or occlusion.

Two placebo-controlled studies were published in the 1990s. The first randomized 199 patients to placebo, 100 mg or 330 mg aspirin (both combined with 75 mg dipyridamole) three times daily and found the difference between placebo and the highest dose of aspirin to be statistically significant[39]. The second study randomized 223 patients to 50 mg of aspirin combined with 400 mg of dipyridamole daily for 3 months or to placebo and found no difference between the two treatment groups[40]. This study included patients with iliac angioplasty (more than 40% of the patients) which has a lower risk of reocclusion.

Later clinical trials evaluated low-dose vs. high-dose aspirin as antithrombotic prophylaxis in conjunction with PTA, and found no difference in late outcome between the two regimen but serious gastrointestinal side effects were fewer with the lower dose of aspirin[41,42]. One study suggested that some patients may require a higher dose of aspirin or alternative antiplatelet agents such as ticlopidine or clopidogrel: all 8 occlusions that occurred in 100 patients treated with 100 mg aspirin for 18 months after an elective PTA occurred in patients with inadequate platelet inhibition on in vitro aggregation testing[43]. Taken together, the data on the effect of aspirin on late restenosis (and reocclusion) are rather disappointing: the conclusion that a low dose of aspirin is as effective as a high dose has little significance (except for the lower complication rate) as long as we do not know whether aspirin has any effect at all on late events. The problem is that new placebo-controlled trials are judged to be unacceptable in view of the effect of aspirin on the general prognosis even though, as discussed above, this effect is poorly documented in patients with peripheral arterial disease. On the other hand, the practice of prescribing aspirin in conjunction with peripheral angioplasty is not seriously challenged either.

Table 71.4. Antiplatelet therapy in angioplasty (and stent placement)

Use aspirin before and after angioplasty of aorto-iliac arteries to reduce incidence of periprocedural thromboembolic events.
Consider aspirin combined with ticlopidine or clopidogrel in patients undergoing angioplasty of femoral and distal arteries.

Note:
Adapted from ref. [10,11].

The proposal to associate ticlopidine (or clopidogrel) to aspirin for procedures below the inguinal ligament is recent. It is entirely based on extrapolation of data after coronary angioplasty: here, the combined use of aspirin and ticlopidine appears to result in synergistic platelet inhibition compared to monotherapy with either agent and in a better early clinical outcome.

Up to now there are no data which specifically address the value of antithrombotic drugs after insertion of stents in peripheral arteries, despite the inherently thrombogenic nature of the material used. Stents in the aorto-iliac region improve the immediate hemodynamic results of iliac angioplasty (particularly in case of complicated lesions and chronic occlusions) and effectively manage recoil and procedure-related flow-limiting dissections[44]. Experienced centres have demonstrated a 1-year and 3-year patency rate after successful recanalization of 90 and 80%, respectively. These results are, in general, obtained with the same antithrombotic regimen as is used in simple angioplasty, although some centres continue heparin administration for 24 to 72 hours.

On the other hand, stenting of infrainguinal lesions as a primary approach is a debated issue: for many, it is at present indicated only for salvage of failed angioplasty due to dissection and marked recoil. Immediate and early

Cambridge Books Online © Cambridge University Press, 2009

results appear satisfactory but intimal hyperplasia in the stented segment in the first 3 to 9 months after treatment is common and often leads to late failure. Would a modification of the antithrombotic strategy improve results for femoropopliteal stents? The combined use of aspirin and ticlopidine (or clopidogrel) is increasingly popular, certainly in European centres with an aggressive policy of primary stenting of femoropopliteal lesions. Again, their practice is based on extrapolation of coronary data. Extrapolation of data from one vascular bed to another assumes an analogous problem and the same cause and underlying mechanism. In the coronary circulation, early stent occlusion is the targeted dramatic event; in the femoropopliteal region, late reocclusion is of greater concern.

Future prospects

The newest, yet unpublished, analysis of the Antithrombotic Trialists' Collaboration[45], mainly confirms the previous one[6], be it on a much larger patient population: antiplatelet therapy reduces the risk of any serious vascular event by roughly one-quarter in all categories of patients at high risk. Is any further progress to be expected from this category of drugs? It is doubtful whether a simple further increase in numbers of patients studied would alter the basic conclusion. The hope that the powerful glycoprotein IIb–IIIa blockers would open a new era of antithrombotic therapy vanished after the debâcle with the oral preparations. A return to the thromboxane receptor blockers is still in an early phase. On the other hand, too many issues in peripheral arterial disease are currently being solved by extrapolation from other vascular beds, particularly the coronary.

References

1 European Working Group on Critical Leg Ischemia. Second European Consensus Document on Chronic Critical Leg Ischemia. *Circulation* 1991; 84 Suppl 4: 1–26.

2 Gresele P, Catalano M, Giammarresi C et al. Platelet activation markers in patients with peripheral arterial disease. A prospective comparison of different platelet function tests. *Thromb Haemost* 1997; 78: 1434–1437.

3 Davi G, Gresele P, Violi F et al. Diabetes mellitus, hypercholesterolemia, and hypertension but not vascular disease per se are associated with persistent platelet activation in vivo. Evidence derived from the study of peripheral arterial disease. *Circulation* 1997; 96: 69–75.

4 Böger RH, Bode-Böger SM, Thiele W, Junker W, Alexander K, Frölich JC. Biochemical evidence for impaired nitric oxide synthesis in patients with peripheral arterial occlusive disease. *Circulation* 1997; 95: 2068–2074.

5 TASC Working Group. Management of peripheral arterial disease: epidemiology, natural history, risk factors. *Inter Angio* 2000; 19 Suppl 1: 5–28.

6 Antiplatelet Trialists' Collaboration. Collaborative Overview of Randomized Trials of Antiplatelet Treatment. Part I: Prevention of death, myocardial infarction and stroke by prolonged antiplatelet therapy in various categories of patients. *Br Med J* 2000; 308: 81–101.

7 Blanchard J, Carreras LO, Kindermans M and the EMATAP-group. Results of EMATAP: a double-blind placebo-controlled multicentre trial of ticlopidine in patients with peripheral arterial disease. *Nouv Rev Fr Hematol* 1993; 35: 523–528.

8 CAPRIE Steering Committee. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischemic events (CAPRIE). *Lancet* 1996; 348: 1329–1339.

9 Girolami B, Bernardi E, Prins MH et al. Antithrombotic drugs in the primary medical management of intermittent claudication: a meta-analysis. *Thromb Haemost* 1999; 81: 715–722.

10 Jackson MR, Clagett GP. Antithrombotic therapy in peripheral arterial occlusive disease. *Chest* 1998; 114: 666S-682S.

11 Verstraete M, Prentice CRM, Samama M, Verhaeghe R on behalf of a European Working Group. A European View on the North American Fifth Consensus on Antithrombotic Therapy. *Chest* 2000; 117: 1755–1770.

12 Schoop W, Levy H, Schoop B, Gaentsch A. Experimentelle und klinische Studien zu der sekundären Prävention der peripheren Arteriosklerose. In Bollinger A, Rhyner K (eds) *Thrombozytenfunktionshemmer*, George Thieme Verlag, Stuttgart, 1983, pp 49–58.

13 Hess H, Mietaschk A, Deichsel G. Drug-induced inhibition of platelet function delays progression of peripheral occlusive arterial disease. A prospective double-blind arteriographically controlled trial. *Lancet* 1985; 1: 415–419.

14 Stiegler H, Hess H, Mietaschk A, Tramppisch HJ, Ingrisch H. Einfluß von Ticlopidin auf die perifere obliterierende Arteriopathie. *Deutsche Medizinische Wochenschrift* 1984; 109: 1240–1243.

15 Goldhaber SZ, Manson JE, Stampfer MJ, LaMotte F, Rosner B, Buring JE, Hennekens CH. Low-dose aspirin and subsequent peripheral arterial surgery in the Physicians' Health Study. *Lancet* 1995; 340: 143–145.

16 Bergqvist D, Almgren B, Dickinson JP. Reduction of requirement for leg vascular surgery during long-term treatment of claudicant patients with ticlopidine: results from the Swedish Ticlopidine Multicentre Study (STIMS). *Eur J Vasc Endovasc Surg* 1995; 10: 69–76.

17 Arcan JC, Panak E. Ticlopidine in the treatment of peripheral occlusive arterial disease. *Semin Thromb Haemost* 1989; 15: 167–171.

18 Boissel JP, Peyrieux JC, Destors JM. Is it possible to reduce the risk of cardiovascular events in subjects suffering from

Cambridge Books Online © Cambridge University Press, 2009

intermittent claudication of the lower limbs *Thromb Haemost* 1989; 62: 681–685.

19 Balsano F, Coccheri S, Libretti A et al. Ticlopidine in the treatment of intermittent claudication: a 21-month double blind trial. *J Laborat Clin Med* 1989; 114: 84–91.

20 Hood SC, Moher D, Barber GG. Management of intermittent claudication with pentoxifylline: meta-analysis of randomized controlled trials. *Can Med Assoc J* 1996; 155: 1053–1059.

21 Comp PC. Treatment of intermittent claudication in peripheral arterial disease. Recent clinical experience with cilostazol. *Today's Ther Trends* 1999; 17: 99–112.

22 Sorkin EM, Markham A. Cilostazol. *Drugs and Aging* 1999; 14: 99–112.

23 Diehm C, Balzer K, Bisler H et al. Efficacy of a new prostaglandin E1 regimen in outpatients with severe intermittent claudication: results of a multicenter placebo-controlled double-blind trial. *J Vasc Surg* 1997; 25: 537–544.

24 Belch JJF, Bell PRF, Creissen D et al. Randomized, placebo-controlled, double-blind study evaluating the efficacy and safety of AS 013, a prostaglandin E1 prodrug, in patients with intermittent claudication. *Circulation* 1997; 95: 2298–2302.

25 Lièvre M, Morand S, Besse B, Fiessinger JN, Boissel JP on behalf of the Beraprost et Claudication Intermittente (BERCI) Research Group. Oral Beraprost sodium, a prostaglandin I2 analogue, for intermittent claudication. A double-blind, randomized, multicenter controlled trial. *Circulation* 2000; 102: 426–431.

26 Tangelder MJD, Lawson JA, Algra A, Eikelboom BC. Systematic review of randomized controlled trials of aspirin and oral anticoagulants in the prevention of graft occlusion and ischemic events after infrainguinal bypass surgery. *J Vasc Surg* 1999; 30: 701–709.

27 Jackson MR, Clagett GP. Antithrombotic therapy in peripheral arterial occlusive disease. *Chest* 2001; 119: 283S-299S.

28 Green RM, Roederscheimer R, De Weese JA. Effects of aspirin and dipyridamole on expanded polytetrafluoroethylene graft patency. *Surgery* 1982; 82: 1016–1026.

29 Goldman M, McCollum C. A prospective randomized study to examine the effect of aspirin plus dipyridamole on the patency of prosthetic femoropopliteal grafts. *Vascular Surgery* 1984; 18: 217–221.

30 Kohler TR, Kaufman JL, Kacoyanis G et al. Effect of aspirin and dipyridamole on the patency of lower extremity bypass grafts. *Surgery* 1984; 96: 462–466.

31 Clyne CAC, Archer TJ, Atuhaire LK, Chant ADB, Webster JHH. Random control trial of a short course of aspirin and dipyridamole (Persantin for femorodistal grafts. *Br J Surg* 1987; 74: 246–248.

32 McCollum C, Alexander C, Kenchington G, Franks PJ, Greenhalgh R. Antiplatelet drugs in femoropopliteal vein bypasses: a multicenter trial. *J Vasc Surg* 1991; 13: 150–162.

33 Becquemin JP, for the Etude de la Ticlopidine après Pontage Fémoro-Poplité and the Association Universitaire de Recherche en Chirurgie. Effect of ticlopidine on the long-term patency of saphenous-vein bypass grafts in the legs. *N Engl J Med* 1997; 337: 1726–1731.

34 Watson HR, Skene AM, Belcher G. Graft material and results of platelet inhibitor trials in peripheral arterial reconstructions: reappraisal of results from a meta-analysis. *Br J Clin Pharmacol* 2000; 49: 479–483.

35 Antiplatelet Trialists' Collaboration. Collaborative overview of randomized trials of antiplatelet treatment. Part III: Maintenance of vascular graft or arterial patency by antiplatelet therapy. *Br Med J* 1994; 309: 159–168.

36 Girolami B, Bernardi E, Prins MH et al. Antiplatelet therapy and other interventions after revascularisation procedures in patients with peripheral arterial disease: a meta-analysis. *Eur J Vasc Endovasc Surg* 2000; 19: 370–380.

37 Sarac TP, Huber TS, Back MR, Ozaki CK, Carlton LM, Flynn TC, Seeger JM. Warfarin improves outcome of infrainguinal vein bypass grafts at high risk for failure. *J Vasc Surg* 1998; 28: 446–457.

38 Dutch Bypass Oral anticoagulants or Aspirin (BOA) Study Group. Efficacy of oral anticoagulants compared with aspirin after infrainguinal bypass surgery (the Dutch Bypass Oral anticoagulants or Aspirin study): a randomised trial. *Lancet* 2000; 355: 346–351.

39 Heiss HW, Just H, Middleton D, Deichsel G. Reocclusion prophylaxis with dipyridamole combined with acetylsalicylic acid following PTA. *Angiology* 1990; 41: 263–269.

40 Study group on pharmacological treatment after PTA. Platelet inhibition with ASA/dipyridamole after percutaneous balloon angioplasty in patients with symptomatic lower limb arterial disease. A prospective double-blind trial. *Eur J Vasc Surg* 1994; 8: 83–88.

41 Ranke C, Creutzig A, Luska G et al. Controlled trial of high- versus low-dose aspirin treatment after percutaneous transluminal angioplasty in patients with peripheral vascular disease. *Clin Invest* 1994; 72: 673–680.

42 Minar E, Ahmadi A, Koppensteiner R et al. Comparison of effects of high-dose and low-dose aspirin on restenosis after femoropopliteal percutaneous transluminal angioplasty. *Circulation* 1995; 91: 2167–2173.

43 Mueller MR, Salat A, Stangl P et al. Variable platelet response to low-dose ASA and the risk of limb deterioration in patients submitted to peripheral arterial angioplasty. *Thromb Haemost* 1997; 78: 1003–1007.

44 TASC Working Group. Management of peripheral arterial disease: treatment of intermittent claudication. *Inter Angio* 2000; 19 Suppl 1: 82–126.

45 Antithrombotic Trialists' (ATT) Collaboration. Prevention of death, myocardial infarction and stroke by antiplatelet therapy: collaborative meta-analysis of 266 trials involving 200,000 patients at high risk of occlusive vascular events. *Br Med J*, submitted

Cambridge Books Online © Cambridge University Press, 2009

Afterword

Platelets: a personal story

Gustav V.R. Born

William Harvey Research Institute, Charterhouse Square, London, UK

Platelets were a major research interest of mine from the 1960s to the 1980s. During that period descriptive knowledge of platelets was superseded by understanding of their pathophysiological mechanisms and functions. The Editors' invitation was for a brief account of personal work, some of which turned out to be seminal, of course in its wider setting. So I hope this will be as enjoyable to read as it was to write. It has made me look again at our platelet publications of so many years ago that I had forgotten much of what we did and was pleasantly surprised by rediscovering it. The request was for a personal story. So the massive and essential contributions from large numbers of contemporary workers are set out in the historical chapter of this book. This account provides another opportunity of acknowledging the many excellent co-workers, listed below, many of whom carried on with research in various parts of the world and some of whom are contributors to this book.

In 1976 the Royal Society invited me to give a lecture to the British Association of Young Scientists. By then platelets had been my major concern for 15 years. So the title of my lecture was *Why don't we Bleed to Death? The vital defence mechanism of hemostasis.* The abstract, made as attractive as possible for young people, still stands as a reliable summary of what platelets are primarily about:

'As soon as primitive metazoan animals acquired a body cavity containing a fluid different in composition from the environmental fluid they had something to defend against change, a *milieu interieur* to keep constant. Any acute injury was liable to cause a change in the composition or amount of the body fluid. For survival it became necessary, therefore, to develop a defence mechanism, the purpose of which was the prevention of loss of body fluid. This defence mechanism is most simply referred to as "hemostasis" although the name should strictly speaking be reserved for animals with blood circulations.

The defence mechanism against the loss of vital body fluid has three components: (i) change in the physical state of the fluid itself which stops it flowing out through a wound; this is the process called clotting or coagulation; (ii) cellular contraction around the wound opening which makes it smaller and so impedes the loss of fluid; and (iii) the adhesion and aggregation of specialised cells in the wound, tending to close it and so to arrest the outflow of fluid.

Human blood contains a particular type of cell, the platelets, the only known physiological function of which is hemostasis. In healthy people this function is in evidence whenever our closed, high-pressure blood-vascular system is opened somewhere through injury, although the contribution of the platelets to the total hemostatic process depends on the type or types of vessels opened up as well as on other conditions.

Pathologically, platelets constitute a major element of arterial thrombi, whether forming rapidly on atherosclerotic vessel walls or more slowly during graft rejection episodes in the supplying vessels. The hemostatic function of platelets is literally 'vital', i.e. essential to survival; and their pathological role is, of course, potentially lethal. This clinical significance has led to a remarkable increase in platelet research from every conceivable aspect. This research seems likely to result, for the first time, in the discovery of drugs for preventing arterial thromboses such as 'acute coronaries' which are the scourge of this and other developed countries.' Note that this was written in 1976, just about the time the most successful antiplatelet drug – Aspirin – did indeed make its début on the heart attack scene.

The beginnings

How does one come to platelets, such a seemingly insignificant non-cell? Since their discovery about 150 years ago the commonest route has been through clinical hematology; and until platelets became prominent players in coronary heart disease and stroke, their medical importance was mainly *in absentia*, because platelet deficiency causes bleeding. There are many causes of platelet deficiency, one being radiation. As an Army Medical Officer posted close to atom-bombed Hiroshima in defeated Japan, deadly hemorrhages from radiation sickness first made me aware of platelets and how much we need them.

But my own long-term relationship with platelets did not come out of disturbing clinical impressions but through my post-doctoral initiation into pharmacological research. In 1956 I was working in the Oxford Pharmacology Department with Hugh Blaschko on the recently discovered association of adrenaline with ATP in adrenal granules, which contain about three molecules of the cationic amine for each molecule of ATP^{3-}, suggesting ionic binding[1]. It occurred to me that other endogenous amines might be stored similarly elsewhere in the body. Somewhere I had heard or read of platelets containing large amount of the vasoconstrictor amine serotonin (5-hydroxytryptamine, 5-HT). So the initial intention was to utilize this, then comparatively unexplored, circulating pseudocell for pharmacological purposes. As the first person in Britain I used the firefly method, recently introduced by McElroy[2] in the United States, for measuring platelet ATP. With the platelet extract adjusted to ATP concentrations expected from other cell types, the galvanometer pointer flew off the scale – an unforgotten moment[3]. The discovery of excess platelet ATP was followed by evidence for its stoichiometric association with 5-HT, again suggesting ionic binding[4–7].

Platelets and aminergic mechanisms

Most of our subsequent work with platelets related to their physiological function in hemostasis and its pathological aberration, thrombosis. But when I began working with platelets they were of interest also through being thought of as biologically rather simpler and therefore easier to study than other types of cell. This has turned out to be a fortunate fallacy: except for the absence of DNA and of protein synthesis, platelets are as complex as the next cell; but because they are easily obtainable almost homogeneously, they have proved invaluable as models for less accessible and more complex cells such as neurones. It was the ability of platelets to be activated by 5-hydroxytryptamine (5-HT) that led to their use as models for tryptaminergic neurones, so different in function and environment, and for determining the modes of action of psychotherapeutic drugs, particularly by Alfred Pletscher and coworkers. One of our own investigations showed that the beneficial effects of lithium in manic-depressives goes with increases in their platelet 5-HT[8]. Another confirmed with rabbits David Boullin's clinical observation that long-term chlorpromazine treatment increases platelet aggregability to 5-HT, possibly through increasing the number of specific receptors[9]. Our evidence suggested that the uptake of 5-HT is an active process[6, 7] and with Oleh Hornykiewicz (who later discovered the dopamine deficiency in Parkinson's disease) that the uptake of adrenaline and noradrenaline is not[10]. As 5-HT induces platelet aggregation, the mechanisms for uptake and aggregation were compared. Using numerous tryptamine analogues it was

Cambridge Books Online © Cambridge University Press, 2009

shown that uptake is highly specific for 5-HT whereas aggregation is not[11]. Intriguingly, uptake was accelerated with platelets covalently enriched with sialic acids on their outer membranes, possibly explained by increased binding of 5-HT at the cell surface[12]. Effects of lithium and trycyclic and other drugs on amine mechanisms demonstrated with platelets were reproduced with nerve cells in the development of antidepressants by pharmaceutical companies.

Platelets in hemostasis and thrombosis

By far the largest and longest part of my work on platelets had to do with their role in hemostasis and thrombosis; more specifically how platelets reduce blood loss from injuries to vessels larger than the capillaries, where they don't; and how platelets come to initiate – and not infrequently dominate – thrombosis in arteries. In both situations, the physiological and the pathological, the fundamental process is that of platelet aggregation, whereby changes in their normal biochemical and/or hemodynamic environment induce individual platelets to adhere to one another and to aggregate into the growing bodies known respectively as hemostatic plugs and arterial thrombi. The evidence that acute coronary thrombosis is almost always initiated by plaque rupture and hemorrhage has disposed of any uncertainty as to why the platelet aggregation process is the same there as in other hemorrhagic situations.

My interest in platelet aggregation was awakened through the coming together of two previously unconnected observations. One was the discovery of excess ATP in platelets and the demonstration of its rapid breakdown in clotting blood[13]. The other was the Norwegians' discovery of the platelet-aggregating effect of ADP[14]. By then aware that the primary function of platelets is their hemostatic aggregation, I suggested that the two processes are connected through the formation of ADP from ATP released from platelets and from other cells involved in vascular injury[15]. This has turned out to be an oversimplification as far as the platelets themselves are concerned, which release ADP mostly from a different pool than that in which ATP breaks down. Nevertheless, over the years the hypothesis that ADP contributes to platelet aggregation in hemostasis and thrombosis has been supported by experiments[16–18], and by clinical trials of drugs which inhibit platelet activation by ADP, notably the CAPRIE Trial published in the *Lancet* as recently as 1998[19]. Subsequent discoveries of other endogenous aggregating agents, predominantly thromboxane A2 and thrombin, have added to the complexity of the process. But the evidence stands that ADP and thromboxane A2 contribute about equally to hemostatic aggregation in vivo[20].

Platelets in vitro: optical aggregometry

For investigations of platelet physiology I invented and developed the optical aggregometer for quantifying and analysing platelet reactions in vitro. The idea came to me from so-called 'turbidimetric' measurements of ribonuclease activity in *Streptomyces* culture filtrates done for my D.Phil. (as the Ph.D. is termed in Oxford which must always be different!). I made the simple adaptations appropriate for measuring platelet aggregation in plasma. This new method brought exciting results quickly and reproducibly. Having had an important part of my scientific upbringing in Professor Joshua Burn's excellent Pharmacology Department in Oxford, where free and noisy communication between members and visitors was strongly encouraged, I had no hesitation in demonstrating the technique immediately to anyone who asked. The method was published in 1962[15,21] and the first detailed description of observations made with it in the following year[22]. We quantified the relation of aggregate formation to the optical changes[23,24] and showed that they could be accounted for on classical light-scattering theory[25].

The optical aggregometer has ever since been used worldwide in fundamental, clinical and epidemiological investigations. Within a few years the original papers became *Citation Classics*; and by January 2001 the *Nature* paper had been cited 3796 times and the *Journal of Physiology* paper 1902 times (P.D. Richardson, personal communication). Many more papers – probably many thousands – have been based on optical aggregometry without reference to the original publications. Commenting on the first *Citation Classic* in 1977, I wrote: 'To be told that this paper is one of the most cited articles ever published is astonishing and interesting. I can think of three possible reasons. First, the paper helped to make platelets interesting research objects by suggesting functions for two, then recent, observations, *viz.* ATP breakdown during clotting and platelet aggregation by ADP. The paper suggests that these processes are connected through the formation of ADP from ATP in cells damaged by or involved in vascular injury . . . much evidence now supports this original proposition.

Secondly, the paper introduced the photometric method which later acquired the horrible name of 'aggregometry'. It soon became widely used, presumably because of its simplicity and reproducibility. It has been responsible for major discoveries in platelet function, notably the second phase of aggregation. This is the optical manifestation of

Cambridge Books Online © Cambridge University Press, 2009

the platelet release reaction, much investigated since as an example of exocytosis and because the discovery of its inhibition by acetyl salicylic acid is the origin of the Aspirin trials in coronary thrombosis.

This brings up the third reason for the popularity of the 1962 paper. It showed that platelet aggregation by ADP can be inhibited and reversed by the closely related substances AMP or ATP. The last paragraph of the paper reads: 'If it can be shown that ADP takes part in the aggregation of platelets in blood vessels it is conceivable that AMP or some other substance could be used to inhibit or reverse platelet aggregation in thrombosis.' Cross and I soon found that AMP was much less inhibitory than adenosine which, in turn, is much less effective than several inhibitors unrelated to ADP which have been discovered since, such as some prostaglandins. Elucidation of these inhibitory mechanisms has made rapid progress, not only because platelets are advantageous models for other cell systems but also because it may result in important advances in drug treatments.' This has indeed turned out to be so.

I have been told there are thousands of optical aggregometers around the world and asked why I did not patent the device. The reason is that before the Thatcherite degradation of everything in life to money, it did not occur to me nor to most members of the medical research community to patent inventions or discoveries; indeed my Oxford Professor Howard Florey was against patenting anything of potential medical value. So the idea just never came up.

As an aside, it occurred to me that the aggregometer might be useful in other biological systems. The life cycle of the slime mould *Dictyostelium discoideum* includes a phase in which thousands of separate cells aggregate into elegant fruiting bodies. So this process was quantified with the aggregometer and the effects of temperature and ionic strengths were determined[26,27]. Interestingly, recalling the platelet effect of lithium referred to earlier, *Dictyostelium* is helping to elucidate the antidepressant action of lithium[28,29].

Up to the molecular biology era, it is probably true to say that much if not most of the new information about platelets has depended on optical aggregometry. Aggregation was characterized with respect to velocity and temperature and pH dependence[22]. Two essential cofactors of aggregation were discovered, viz., calcium and fibrinogen[30,31]; and the suggestion that fibrinogen forms 'bridges' linking aggregating platelets[32] was later confirmed at the electromiscroscopic and molecular levels. Nowadays this bridging function, and thereby platelet aggregation, can be prevented by various molecules which inhibit binding of fibrinogen to its receptor, the activated conformer of Glycoprotein IIb–IIIa, on the platelet surface. So far, meta-analyses of clinical trials indicate that these agents given orally do not decrease and may even increase mortality, whereas given intravenously they improve the outcome of coronary stents[33,34].

The rapid shape change of platelets (time constant about 1 second at 37 °C) which is the first visual evidence of their activation was quantified and shown to conform to Michaelis–Menten kinetics[35]. The results suggested that aggregation agonists such as ADP react with specific membrane receptors leading to structural changes; now of course the receptors are fully identified. The same paper contains the proof, by means of a novel thrombocrit technique for determining cell volumes directly, that the rapid shape change is not accompanied by an increase in mean platelet volume, as had been widely accepted on the basis of Coulter Counter measurements. This finding threw doubt on the validity of cell volume measurements by such widely used indirect methods.

Optical aggregometry made possible the discovery by Macmillan and Oliver in 1963 of the second phase of aggregation[36] – Michael Cross and I had failed to notice or to think about the anomalous deformations in our manually plotted aggregometer tracings (this was before the availability of continuous recording) which turned out to be the optical manifestation of the platelet release reaction. In this reaction, enough ADP is released[37] to account for the positive feedback mechanism of aggregate growth already proposed. It is this second phase of aggregation which, as discovered in 1967 by Mustard and coworkers[38] and by Harvey Weiss and Louis Aledort[39], is inhibited by Aspirin. Therefore optical aggregometry is at the beginning of a very important medical story. Ever since I have enjoined my students to look for odd or unexpected findings and never to disregard what could be an experimental error but could also be something real and important. It was a salutary lesson, in principle – but only in principle – analogous to the unexpected and curious astronomical tracings obtained by S.J. Bell and A. Hewish which led to their discovery of the pulsars. The feedback hypothesis has received much experimental support. Feedback is promoted by adrenaline[40] and inhibited by drugs which prevent the release of ADP from platelets.

Optical aggregometry soon led also to the discovery of the first aggregation inhibitors, viz., ATP and adenosine, looked at first because of their close chemical relationship to pro-aggregatory ADP[15,41]. As it has turned out, inhibition was the most exciting and far-reaching discovery because it established the therapeutic possibility of preventing arterial thrombosis by means of antiplatelet drugs and initiated the era of their use for the prevention of myocardial infarction and stroke. Much effort was devoted to inhibitory mechanisms. We showed that ATP inhibited competitively whereas adenosine did not but was significantly more

Cambridge Books Online © Cambridge University Press, 2009

effective[42]. More effective still were the 2-substituted adenosine derivatives, particularly 2-chloroadenosine, out of many related substances[43]. In experiments on ourselves, which could conceivably have been harmful and which would now be strictly forbidden, we showed that the relative effectiveness of adenosine analogues as aggregation inhibitors and as arterial vasodilators was the same[44]. Since then this has been explained by the similarity of the cyclic AMP mechanism in platelets and in vascular smooth muscle. We showed later that ATP as a competitive inhibitor of aggregation by ADP is able to reverse the rapid shape change of platelets, bringing them back to their normal disc-like appearance. To demonstrate this rigorously required correlating optical aggregometry with quantitative electron microscopy[45]. Noel Cusack and Susanna Hourani developed nucleosides, nucleotides and several synthetic analogues into important pharmacological tools for investigating platelet mechanisms[46]. By now, three types of receptors for extracellular nucleotides (P2 purinoceptors) are recognized as participants in platelet activation[47].

The drug dipyridamole was shown to inhibit the uptake of adenosine by platelets and to potentiate its inhibitory effects on aggregation[48]. So, as an aside, when Dinah James, Professor of Pharmacology in Ibadan and an authority on African sleeping sickness, joined me for a year and told me that the causative trypanosome cannot synthesize nucleotides *de novo* but only from adenosine taken up from the surrounding tissue fluid, we looked at dipyridamole as an uptake inhibitor[49]. I do not know whether this idea has been taken further – probably not, or I would have heard of it.

Platelets in vivo: hemostatic and thrombotic mechanism

From the time of Bizzozero (1882) it has been known that bleeding from small vessels induces platelets to aggregate before clotting begins. Our first question was whether ADP is involved in activating platelets in vivo as had been shown in vitro. Novel techniques were developed for reproducible bleeding time determinations from small arteries in the superior mesenteric territory of rats and rabbits. In both species, local infusions of active but not of inactive ADP-removing enzyme systems increased bleeding time significantly, supporting the conclusion that platelet aggregation in primary hemostasis involves ADP[16,17]. In rats these bleeding times were also prolonged by ADP receptor antagonists[50] (– this et al. includes a great-granddaughter of Charles Darwin!). With ultra-sensitive luminescence measurements blood at arterial puncture sites was shown to contain micromolar ATP for at least 2 seconds[51,52]. Its rapid dephosphorylation supported the proposition[21] that ADP is involved in initiating hemostatic platelet aggregation[15].

Quantifying platelet adhesiveness and aggregation in uninjured vessels required a methodological innovation. This was to apply ADP by micro-iontophoresis to the outsides of small blood vessels[53]. The technique permitted highly localized measurements of small vessel reactions to pharmacological agents, thereby minimizing other tissue and blood flow effects. ADP thus applied showed that platelet thrombi can be made to form many times on the same site in small blood vessels without damaging their endothelial lining, thereby disposing of claims to the contrary. Aggregates grew exponentially, the rate constant providing a measure of the effects of blood flow and of inhibitors[54]. The technique also permitted a first determination of platelet activation time (of the order of 100 ms, since revised downwards)[55]. Histamine, which has no direct effect on platelets, applied iontophoretically to venules contracts their endothelial cells[56] and reversibly accelerated intravascular platelet aggregation by iontophoretic ADP at the same site, evidence that effects within blood vessels of extravascular agents may be influenced by endothelial contractility[57]. Incidentally, the technique also permitted quantification of the reversible contractility of endothelial cells in vivo and determination of their 'relaxation time'. Platelet–endothelial interactions were shown to be minimal in normal vessels, with only a small proportion adhering for very short times (less than 1 second)[58]. On the other hand, reduction of endothelial negative surface charge greatly increased platelet adhesion[59].

As an aside, we showed that the negative charge densities on endothelia in different blood vessel types greatly exceed those on the surfaces of other cells[60]. This suggested a physiological answer to the long-puzzling question, which had been considered theoretically by Sir James Lighthill, how the blood manages to flow through capillaries with diameters smaller than those of the circulating cells. Our experiments gave evidence that microvascular blood flow is made possible by electrostatic repulsion between the negative charges on endothelial and red cell surfaces[61]. (I often think of this work, which in a sense continues Harvey's and Malpighi's discoveries of how the blood circulates, as the most interesting I have done!)

Overall our findings do little to support the claims of functionally significant interchanges of prostaglandin precursors between platelets and vessel walls, nor of the so-called prostacyclin–thromboxane balance hypothesis[62] which claimed that these two mutually antagonistic agents play a major role in acute coronary thrombosis. The hypothesis, attractive by being simple, was accepted uncritically for many years. But every medical student sees

Cambridge Books Online © Cambridge University Press, 2009

for himself in the postmortem room the gross damage caused by coronary plaque rupture, which makes it inconceivable that these evanescent agents could possibly have any significant effects on the overwhelming thrombogenetic sequelae[63]. The lesson is to stay in touch with real life or, in this case, real death.

As it has turned out, great importance attached to the demonstration that inhibitors of in vitro aggregation are also effective in vivo. The first paper on this[64] showed that the formation and embolization of platelet thrombi is inhibited by adenosine and 2-chloroadenosine. Regional administration of aggregation inhibitors prevented thrombus formation in artificial organs[65].

During the years devoted mainly to platelets, work was also done on other blood cells. The red cells are a source of proaggregatory ADP, as first demonstrated by the Norwegians[14]. We produced evidence that intravascular platelet aggregation is inhibited by chlorpromazine and similar drugs through an action on the red cells rather than on the platelet themselves[66], an effect depending on properties of the erythrocyte membrane[67]. Interactions between red cells and platelets were analyzed under various flow conditions[68].

Platelets in humans

Great advances in the understanding and the therapeutic control of platelets came in the 1970s and 1980s, which were the heyday of the prostaglandins. For platelet people the most important discoveries were proaggregatory thromboxane A2 produced by platelets and antiaggregatory prostacyclin produced by vessel walls; and of course the Nobel Prize-winning discovery by John Vane of the action of Aspirin in inhibiting prostaglandin biosynthesis, plus the immediate demonstration of its applicability to platelets by our graduate students Bryan Smith and Jim Willis. These events are very well known, and the therapeutic advances they brought with them are very great: through its antiplatelet action Aspirin reduces the risk of heart attacks and strokes by up to 40%. Based on evidence that thromboxane A2 and ADP contribute about equally to thrombotic platelet aggregation, large-scale clinical trials are now under way in which Aspirin as thromboxane A2 inhibitor together with Clopidogrel as ADP antagonist are administered together, against Aspirin itself. The results should show whether inhibiting two different platelet activating mechanisms is clinically more effective than inhibiting only one. (Note added in proof: it is!)

Also during that period great interest began in the influence of diet on atherosclerosis and its thrombotic complications. Again, much of this turned on prostaglandins and their precursors. Animal and human evidence, for example from Greenland Eskimos, had established that diets rich in polyunsaturated marine lipids, particularly eicosapentaenoic acid, increased blood loss from injuries and from clinical bleeding time measurements. (The late Hugh Sinclair of Magdalen College Oxford took the diet to such extremes that his bleeding time became virtually infinite!) These effects were generally attributed to a decrease of thromboxane A2 production by platelets demonstrable in clotting blood. Joined by Margaretha Thorngren, who had already been experimenting on her fish-fed husband, we muddied this seemingly clear explanatory pool. Techniques were devised for measuring thromboxane A2 in the blood coming from standardized bleeding time incisions, just where the aggregating platelets should be releasing it. Compared to blood clotting in vitro bleeding time blood contained very little thromboxane A2, suggesting that it is not a major determinant of that parameter[69]. Secondly, volunteers on a fish diet for 4 weeks had the expected increase in bleeding time and decrease in clotted blood thromboxane A2 but no diminution in its output in bleeding time blood[70]. Our evidence also indicated that the bleeding time increases produced by fish diets and by Aspirin are brought about by different mechanisms[71].

So we looked into another possible explanation, namely that diet diminishes the local vasoconstriction caused by injuries such as cutting. We found that in rats on a diet enriched in eicosapentaeonic acid the contractile effect on small mesenteric arteries of noradrenaline as the primary endogenous vasoconstrictor was decreased up to fourfold[72]. It all adds up to the conclusion that the increased hemorrhagic tendency of fish-eating populations has less to do with platelets than with vascular contractility – at the time an heretical proposition which may however have been followed up more than I am aware of.

Envoi

By the mid-1980s, understanding of the physiological behaviour and pharmacologial control of platelets was well advanced. New questions were increasingly answered by the techniques of molecular biology, with which I am not familiar. So it seemed natural to turn for my last research decade to atherosclerosis, particularly because, to the extent that it becomes preventable, the clinical importance of thrombosis and therewith of platelets will diminish. Many years before we had demonstrated experimentally that lipemia accelerates the growth of

Cambridge Books Online © Cambridge University Press, 2009

platelet thrombi in vivo[73] establishing a link between thrombogenesis and atherogenesis. Work by many people has elucidated the interactive contributions of plasma lipids and platelets to arterial thrombotic events. The early finding has been refined by showing that the aggregability of platelets may be significantly increased by low density lipoprotein (LDL)[74]. And so this vascular journey continued in opposite directions – upstream towards atherogenesis by investigating factors which determine the flux of atherogenic plasma proteins from the blood into arterial walls; and downstream to the terminating event of plaque fissure, the immediate cause of coronary thrombosis in which platelets are heavily involved. With two outstanding colleagues, the pathologist Michael Davies and the physicist Peter Richardson, the first quantification was made of the roles of plaque configuration, stress distribution and macrophage density as determinants of plaque rupture[75]. Plaque fissure represents the link between atherosclerosis and thrombosis[63]. Therefore, why plaques rupture and how that may be prevented are questions of clear and present concern to the platelet community.

Looking back, I suppose that the greatest change to have happened to platelets is their enormously increased importance in clinical medicine – from somehow being involved in the rather uncommon conditions of idiopathic thrombocytopenia and radiation sickness to being recognized as a dominant factor in arterial thromboses causing myocardial infarction and stroke, which together cause almost half of all deaths in industrialized populations. Surprisingly, the exact mechanism whereby platelet deficiency is associated with petechial bleeding is still uncertain. In contrast, the mechanism of platelet aggregation at vessel injuries is now well understood. It is gratifying that the feedback hypothesis of platelet aggregation turned out to be explanatory of the remarkable effectiveness of antiplatelet drugs of the Aspirin type in the prevention of heart attacks and strokes. The continuing medical importance of platelets is attested by the development of antiplatelet agents with other modes of action, some already in use and others undergoing clinical trials[33]; and by the existence of the Anti-Platelet Trialist Organization in Oxford which has been monitoring progress worldwide.

Thought-provoking developments in the platelet story keep me interested and involved; for example, by helping to evaluate the comparative clinical merits of Aspirin and Clopidogrel[76]. And recently there was the unexpected and curious result of the Thrombosis Prevention Trial[77], that non-fatal events were significantly reduced by Aspirin but not by Warfarin which however significantly reduced fatal events. If substantiated this finding cries out for a reasonable, perhaps even testable, hypothesis. I suggested that the simplest assumption might be differences in fissure size, such that non-fatal events were associated with small fissures inducing non-occlusive embolizing platelet thrombi, rather like unstable angina, whereas fatal events would be caused by large fissures where platelet aggregation is rapidly superseded by occlusive coagulation, so accounting for the great effectiveness of Warfarin. Perhaps it is conceivable that further improvements in high-resolution ultrasonography or magnetic resonance imaging techniques might show up differences in fissure size, at least in carotid arteries[78].

Platelet research has been continuously interesting itself and has also permitted following up promising side alleys. It would be a pity if career pressures on research workers were to prevent them from following up ideas not on their main road. I myself have still been lucky enough to do that – the three or four ‘asides’ referred to above were not the only ones. Of course there was a downside: to quote from my graduation anniversary, ‘years on minimal income at the beginning, and not much more at the end; but an amazing amount of fun’. And now, late in the day, there beckons the possibility of following up an exciting idea in a different field altogether, namely cancer. But that is another story. At all events, I am glad to have contributed in a small way to knowledge of a vital physiological process – hemostasis – and to an advance in Medicine, which Dr Samuel Johnson rightly called ‘the greatest benefit to mankind’.

In the platelet work the following were my principal colleagues and coworkers, whose contributions and friendship I wish to acknowledge with pleasure and gratitude:

Karl Afors: Sweden
Mara Baldacci: Italy
Massímo Baldacci: Italy
Hans Baumgartner: Switzerland
Nicola Begent: UK
David Bergquist: Sweden
Robert Berne: USA
The late Michael Cross: UK
Noel Cusack: New Zealand
Michael Davies: UK
Peter Esnouf: UK
The late Harold Feinberg: USA
James Foulks: Canada
Peter Görög: Hungary
Richard Haslam: UK
Oleh Hornykiewicz: Austria
Susanna Hourani: UK
Kanchana Juengjaroen: Thailand
Hanno Kortenhaus: Germany
Iren Kovacs: Hungary
Michael Kratzer: Germany
Paul Latimer: USA
Myra McClure: UK
Frank Michal: Australia
David Mills: UK
Wulf Palinski: Germany
Carlo Patrono: Italy
The late Helen Payling-Wright: UK
Richard Philp: Canada
Peter Richardson: USA
Gordon Roberts: UK
Francis Robicsek: USA
Ingrid Schraufstätter: Germany
Shahida Shafi: UK
Bryan Smith: UK
Duncan Thomas: UK
Margaretha Thorngren: Sweden
Artur Wehmeier: Germany
Krystina Zawilska: Poland

Cambridge Books Online © Cambridge University Press, 2009

References

1 Blaschko H, Born GVR, D'Iorio A, Eade NR. Observations on the distribution of catecholamines and adenosine triphosphate in the bovine adrenal medulla. *J Physiol* 1956; 133: 548–557.

2 Strehler BC, Totter JR. Determination of ATP and related compounds: firefly luminescence and other methods. In Glick D (ed.) *Methods of biochemical analysis* London: Interscience. 1954: Vol.1, 341–356.

3 Born GVR. Adenosine triphosphate (ATP) in blood platelets. *Biochem J* 1956a; 62: 33P.

4 Born GVR, Ingram GIC, Stacey RS. The proportionality between the amounts of 5-hydroxytryptamine and adenosine triphosphate in blood platelets. *J Physiol* 1956; 135: 63–65.

5 Born GVR, Ingram GIC, Stacey RS. The relationship between 5-hydroxytryptamine and adenosine triphosphate in blood platelets. *Br J Pharmacol Chemother* 1958; 13: 62–64.

6 Born GVR, Gillson RE. Studies on the uptake of 5-hydroxytryptamine by blood platelets. *J Physiol* 1959; 146: 472–491.

7 Born GVR, Bricknell J. The uptake of 5-hydroxytryptamine by blood platelets in the cold. *J Physiol* 1959; 147: 153–161.

8 Born GVR, Grignani G. Martin K. Long-term effect of lithium on the uptake of 5-hydroxytryptamine by human platelets. *Br J Clin Pharmacol* 1980; 9: 321–325.

9 Born GVR. Increases in aggregation by and uptake of 5-hydroxytryptamine with platelets from rabbits treated with chlorpromazine. *Br J Pharmacol* 1980; 69: 113–118.

10 Born GVR, Hornykiewicz O, Stafford A. The uptake of adrenaline and noradrenaline by blood platelets of the pig. *Br J Pharmacol Chemother* 1958; 13: 411–414.

11 Born GVR, Juengjaroen K, Michal F. Relative activities on and uptake by human blood platelets of 5-hydroxytryptamine and several analogues. *Br J Pharmacol* 1972; 44: 117–139.

12 Szabados L, Mester L, Michal F, Born GVR. Accelerated uptake of 5-hydroxytryptamine by human blood platelets enriched in sialic acid. Short Communication. *Biochem J* 1975; 148: 335–336.

13 Born GVR. The break-down of adenosine triphosphate in blood platelets during clotting. *J Physiol* 1956b; 133: 61–62.

14 Gaarder A, Jonssen J, Laland S, Hellem A, Owren PA. Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. *Nature (Lond)* 1961; 192: 531–532.

15 Born GVR. Aggregation of blood platelets by adenosine/diphosphate and its reversal. *Nature (Lond)* 1962b; 194: 927–929.

16 Begent NA, Zawilska M, Born GVR. Effect of ADP-utilising enzymes of the arterial bleeding time in rats and rabbits. *Br J Haematol* 1982; 50: 317–325.

17 Begent NA, Born GVR. Further evidence that haemostasis depends more on ADP than on thromboxane A_2 in the rat. *J Physiol* 1983; 34: 36P.

18 McClure MO, Kakkar A, Cusack NJ, Born GVR. Evidence for the dependence of arterial haemostasis on ADP. *Proc Roy Soc London B* 1988; 234: 255–262.

19 CAPRIE Steering Committee. A randomized, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). *Lancet* 1996; 348: 1329–1339.

20 Born GVR. Adenosine diphosphate as a mediator of platelet aggregation *in vivo*: an editorial view. *Circulation* 1985; 72: 741.

21 Born GVR. Quantitative investigations into the aggregation of blood platelets. *J Physiol* 1962a; 162: 67–68.

22 Born GVR, Cross MJ. The aggregation of blood platelets. *J Physiol* 1963a; 168: 179–195.

23 Born GVR, Hume M. Effects of the numbers and sizes of platelet aggregates on the optical density of plasma. *Nature (Lond)* 1967; 215: 1027–1029.

24 Michal F, Born GVR. Effect of the rapid shape change of platelets on the transmission and scattering of light through plasma. *Nature (New Biol)* 1971; 231: 220–222.

25 Latimer P, Born GVR, Michal F. Application of light-scattering theory to the optical effects associated with the morphology of blood platelets. *Arch Biochem Biophys* 1977; 180: 151–159.

26 Born GVR, Garrod D. Photometric demonstration of aggregation of slime mould cells showing effects of temperature and ionic strength. *Nature (Lond)* 1968; 220: 616–619.

27 Garrod DR, Born GVR. Effect of temperature on the mutual adhesion of pre-aggregation cells of the slime mould. *Dictyostelium discoideum*. *J Cell Sci* 1971; 8: 751–765.

28 Robin SB, William J, Harwood AJ. *Trends Pharmacol Sci* 2000; 21: 61–64.

29 *Wellcome News*. Drugs, depression and *Dictyostelium*. 1999; Q4: 24–25.

30 Born GVR, Cross MJ. Effects of inorganic ions and of plasma proteins on the aggregation of blood platelets by adenosine diphosphate. *J Physiol* 1964; 170: 397–414.

31 Cross MJ. Effect of fibrinogen on the aggregation of platelets by adenosine diphosphate. *Thromb Diath Haemorrh* 1964; 12: 524–527.

32 Born GVR. Platelets in thrombogenesis: mechanisms and inhibition of platelet aggregation. *Ann R Coll Surg Engl* 1965; 36: 200–206.

33 Bhatt DL, Topol EJ. Current role of platelet glycoprotein IIb/IIIa inhibitors in acute coronary syndromes. *JAMA* 2000; 284: 1549–1558.

34 Chew DP, Bhatt DL, Sapp S, Topol EJ. Increased mortality with oral platelet glycoprotein IIb/IIIa antagonists. A meta-analysis of phase III multicenter randomized trials. *Circulation* 2001; 103: 201–206.

35 Born GVR. Observations on the change in shape of blood platelets brought about by adenosine diphosphate. *J Physiol* 1970; 209: 487–511.

36 Macmillan DC, Oliver MF. The initial changes in platelet morphology following the addition of adenosine diphosphate. *J Atheroscler Res* 1965; 5: 440–444.

37 Mills DCB, Rob IA, Roberts GCK. The release on nucleotides, 5-hydroxytryptamine and enzymes from human blood platelets during aggregation. *J Physiol* 1968; 195: 715–729.

38 Evans G, Packham MA, Nishizawa EE, Mustard JF, Murphy EA. The effect of acetylsalicylic acid on platelet function. *J Exp Med* 1968; 128: 877–894.

Cambridge Books Online © Cambridge University Press, 2009

39 Weiss HJ, Aledort LM. Impaired platelet-connective-tissue reaction in man after aspirin ingestion. *Lancet* 1967; 2: 495–497.

40 Born GVR, Mills DCB, Roberts GCK. Potentiation of platelet aggregation by adrenaline. *J Physiol* 1967; 191: 43–44P.

41 Clayton S, Born GVR, Cross MJ. Inhibition of the aggregation of blood platelets by nucleotides. *Nature (Lond)* 1963; 200: 138–139.

42 Born GVR, Cross MJ. Inhibition of the aggregation of blood platelets by substances related to adenosine diphosphate. *J Physiol* 1962; 166: 29–30P.

43 Born GVR. Strong inhibition by 2-chloroadenosine of the aggregation of blood platelets by adenosine diphosphate. *Nature (Lond)* 1964; 202: 95–96.

44 Born GVR, Haslam RJ, Goldman M, Lowe RD. Comparative effectiveness of adenosine analogues as inhibitors of blood platelet aggregation and as vasodilators in man. *Nature (Lond)* 1965; 205: 678–680.

45 Born GVR, Dearnley R, Foulks JG, Sharp DE. Quantification of the morphological reaction of platelets to aggregating agents and of its reversal by aggregation inhibitors. *J Physiol* 1978; 280: 193–212.

46 Hourani SMO, Cusack NJ. Pharmacological receptors on blood platelets. *Pharmacol Revs* 1991; 43: 243–298.

47 Kunapuli SP. Multiple P2 receptor subtypes on platelets: a new interpretation of their function. *Trends Pharmacol Sci* 1998; 19: 391–394.

48 Born GVR, Mills DCB. Potentiation of the inhibiting effect of adenosine by drugs that prevent its uptake. *J Physiol* 1969; 202: 41–42.

49 James DM, Born GVR. Uptake of purine bases and nucleotides in African trypanosomes. *Parasitology* 1980; 81: 383–393.

50 Born GVR, Cusack HJ, Darwin C, McClure M. Prolongation of bleeding time in rats by ADP receptor antagonists. *J Physiol* 1986; 381: 22P.

51 Born GVR, Kratzer MAA. Source and concentration of extracellular adenosine triphosphate during haemostasis in rats, rabbits and man. *J Physiol* 1984; 354: 419–429.

52 Kratzer MAA, Born GVR. Simulation of primary haemostasis in vitro. *Haemostasis* 1985; 15: 357–362.

53 Duling BR, Berne RM, Born GVR. Micro-iontophoretic application of vasoactive agents to the microcirculation of the hamster cheek pouch. *Microvasc Res* 1968; 1: 158–173.

54 Begent NA, Born GVR. Growth rate *in vivo* of platelet thrombi, produced by iontophoresis of ADP, as a function of mean blood flow velocity. *Nature (Lond)* 1970; 227: 926–930.

55 Born GVR, Richardson PD. Activation time of blood platelets. *J Membrane Biol* 1980; 57: 87–90.

56 Majno J, Shea SM, Leventhal M. Endothelial contraction as a mechanism of action of histamine-type mediators. An electron microscopic study. *J Cell Biol* 1969; 42: 647–672.

57 Begent NA, Born GVR, Sharp DE. The initiation of platelet thrombi in normal venules and its acceleration by histamine. *Physiology* 1972; 223: 229–242.

58 Born GVR, Kortenhaus H, Schroer H. Quantification of the adhesion of platelets in hamster venules *in vivo*. *Proc Roy Soc Lond B* 1982; 215: 135–145.

59 Görög P, Schraufstätter I, Born GVR. Effect of removing sialic acids from endothelium on the adherence of circulating platelets in arteries in vivo. *Proc Roy Soc Lond B* 1982; 214: 471–480.

60 Born GVR, Palinski W. Unusually high concentrations of sialic acids on the surface of vascular endothelia. *Br J Exp Path* 1985; 66: 543–549.

61 Born GVR, Palinski W. Increased microvascular resistance to blood flow in the rat after perfusion with neuraminidase. *J Physiol* 1989; 419: 169–176.

62 Bunting S, Moncada S, Vane JR. The prostacyclin-thromboxane A_2 balance: pathophysiological and therapeutic implications. *Br Med Bull* 1983; 39: 271–276.

63 Lendon C, Born GVR, Davies MJ, Richardson PD. Plaque fissure: the link between atherosclerosis and thrombosis. *Nouv Rev Fran d'Hématol* 1992; 34: 27–29.

64 Born GVR, Honour AJ, Mitchell JRA. Inhibition by adenosine and by 2-chloroadenosine of the formation and embolization of platelet thrombi. *Nature (Lond)* 1964; 202: 761–765.

65 Richardson PD, Galletti PM, Born GVR. Regional administration of drugs to control thrombosis in artificial organs. *Trans Amer Soc Artif Int Organs* 1976; 22:22–29.

66 Born GVR, Wehmeier A. Inhibition of platelet thrombus formation by chlorpromazine acting to diminish haemolysis. *Nature (Lond)* 1979; 282: 212–213.

67 Born GVR, Housley G. Influence of charge and molecular size on membrane stabilisation. *Proc Roy Soc Lond B* 1983; 219: 83–90.

68 Schmid-Schönbein H, Born GVR, Richardson PD, Cusack N, Rieger H, Forst R, Rohling-Winkel P, Blasberg P, Wehmeier A. Rheology of thrombotic processes in flow: the interaction of erythrocytes and thrombocytes subject to high flow forces. *Biorheol* 1981; 18: 415–444.

69 Thorngren M, Shafi S, Born GVR. Thromboxane A_2 in skin-bleeding time blood and in clotted venous blood before and after administration of acetylsalicylic acid. *Lancet* 1983a; I: 8333.

70 Thorngren M, Shafri S, Born GVR. Delay in primary haemostasis produced by a fish diet without change in local thromboxane A_2. *Br J Haematol* 1984; 58: 567–578.

71 Thorngren M, Shafi S, Born GVR. Quantification of blood from skin bleeding time determinations: effects of fish diet or acetylsalicylic acid. *Haemostasis* 1983b; 13: 282–287.

72 Begent NA, Shafi S, Thorngren M, Born GVR. Increased bleeding time associated with decreased vascular contractility in rats fed polyunsaturated lipids. *J Physiol* 1984; 349: 69P.

73 Born GVR, Philp RB. Effects of adenosine analogues and of heparin in non-lipaemic and lipaemic rats. *Br J Exp Path* 1965; 46: 569–576.

74 Bruckdorfer KR. Lipoproteins and platelets. In Betteridge J, Ilingworth R, Shepherd J (eds). *Lipoproteins in health and disease.* Edward Arnold 1999: 619–643.

75 Richardson PD, Davies MJ, Born GVR. The role of plaque configuration and stress distribution in the pathogenesis of

Cambridge Books Online © Cambridge University Press, 2009

fissuring in human coronary atherosclerotic plaques. *Lancet* 1989; ii: 941–944.
76 Gorelick B, Born GVR, D'Agostino RB, Hanley Jr DF, Moye L, Pepine CJ. Therapeutic benefit. Aspirin revisited in light of the introduction of Clopidogrel. *Stroke* 1999; 30: 1716–1721.
77 The Medical Research Council's General Practice Research Framework. Thrombosis prevention trial: randomised trial of low-density oral anticoagulation with warfarin and low-dose aspirin in the primary prevention of ischaemic heart disease in men at increased risk. *Lancet* 1998; 351: 233–241.
78 Born GVR. Effects of aspirin and warfarin on fatal and non-fatal heart attacks. *Lancet* 1999; 354: 1472.

Cambridge Books Online © Cambridge University Press, 2009

Index

abciximab, 422, 588–9, 691, 943–4, 965
  acute coronary syndrome therapy
    non-ST elevation, 1019
    ST elevation, 1022–4
  genetic variability in response, 970
  induced thrombocytopenia, 587, 590–1
  integrin crossreactivity, 945–6
  LIBS epitope expression and, 946
  percutaneous coronary intervention and, 1025–31, 1032
  pseudothrombocytopenia and, 589
  see also GPIIb–IIIa antagonists
angiotensin-converting enzyme inhibitors, 717
acetylsalicylic acid, 919
  see also aspirin
acid hydrolases, 109, 110, 405
acquired amegakaryocytic thrombocytopenic purpura (AATP), 532
actin, 14, 93
actin filaments, 46, 58–9
  assembly, 93, 95, 396
    nucleation by WASp and Arp2/3 complex, 98
    platelet activation and, 95–8
    protein kinase C and, 275
    regulation, 96–8
  discoid shape maintenance role, 57–8, 94
  dynamics in resting platelet, 93–4
  phospholipase C and, 248–9
  platelet shape change and, 326–8
  pleckstrin effects, 276–7
  store-mediated calcium entry and, 263–5, 266
  stress fibres, 395, 397
actin-binding protein (ABP), 296–7
acute autoimmune thrombocytopenic purpura (AITP) in
      childhood, 557, 558–61
  therapy, 559–60
acute coronary syndromes (ACS), 727, 728, 734, 742–3, 1039
  antiplatelet therapy
    non-ST elevation ACS, 1017–19
    secondary prevention, 1015
    ST elevation ACS, 1019–24
  see also specific conditions

Cambridge Books Online © Cambridge University Press, 2009

acute leukemia (AL), 536
  acute lymphoblastic leukemia, 536
  acute myeloid leukemia (AML), 532, 533, 536, 696
    acute promyelocytic leukemia (APML), 536
    familial platelet disorder with predisposition to AML (FPD/AML), 522
    myelodysplastic syndrome and, 534, 535
adducin, 93, 94, 95, 97–8
  platelet shape change and, 328
adenosine, 290, 292, 856, 1064
  inhibition of platelet aggregation, 348
  migraine and, 900
  see also ADP; ATP
adenosine receptors, 290, 292
adenylyl cyclase, 114, 146, 293
  activation, 290–2
    adenosine receptors, 292
    DP receptor, 292
    EP2 receptor, 290–2
    EP4 receptor, 292
    IP receptor, 290
  G protein interaction, 206
    inhibitory G-protein (Gi), 209–10
    stimulatory G-protein (Gs), 207–9
  inhibition, 127, 128, 133, 206, 292–3
    ADP, 292, 343, 973
    EP3 receptor, 292
    epinephrine receptor, 293
    thrombin receptor, 293
    thromboxane receptor, 293
adhesion *see* cell adhesion; platelet adhesion
adhesion receptors *see* platelet receptors
ADP, 4, 8–9, 113–14, 127, 1064–5
  adenylyl cyclase and, 343
  antiplatelet therapy monitoring, 472
  calcium entry and, 261–2, 930
  effects on platelets, 127–8, 209, 929–30
    aggregation induction, 133–5, 342–3, 444, 462, 929, 1064–5
    inhibition of effects, 929, 930, 932
    tumour cell effects, 826
  phospholipase C activation, 239, 244–5
  phospholipase D activation, 252
  release of, 358
  uremia and, 838
ADP receptor antagonists, 131, 135, 473, 930, 932
  acute coronary syndrome therapy
    non-ST elevation, 1017
    ST elevation, 1022
  percutaneous coronary intervention and, 1024–5
  primary prevention, 1013
  secondary prevention, 1014–15
  see also clopidogrel; ticlopidine
ADP receptors, 128–35, 664, 973
  adenylyl cyclase inhibition, 292, 973
  agonists, 131
  congenital defects, 133, 358, 664–5, 678
  molecular identity, 128–9
  pharmacology, 131–3
    clopidogrel and, 131–3, 135
    ticlopidine and, 131, 135
  role in platelet function, 133–5, 209–10, 342, 930
  see also ADP receptor antagonists; P2X receptor; P2Y receptors
adrenaline *see* epinephrine
adrenoceptors, 898–9
adult respiratory distress syndrome, 870
advanced glycation end products (AGEs), 745
afibrinogenemia, 683
  therapy, 683
ageing, of platelets, 71
agglutination
  botrocetin-induced, 348
  ristocetin-induced, 348, 463
  see also platelet aggregation
aggregation *see* platelet aggregation
aggregometry, 462–3, 474, 1063–4
  antiplatelet therapy monitoring, 473–4
aggression, 900
aggretin, 165
AKT protein kinase, 436
albolabrin, 831
albumin, 887–8, 889
alcohol abuse, 293, 902
  thrombocytopenia and, 531
all-*trans* retinoic acid (ATRA), 536
allergic diseases, 852–61
  IgE role in platelet function, 854–5
  platelet involvement, 852–4
    animal models, 855–6
  see also asthma
allergic reactions to platelet transfusion, 717–18
alloimmune thrombocytopenia, 551–2, 561–2, 569–80, 694
  antigen testing, 575–6
  diagnosis, 574
  human platelet-specific antigens, 570–1
  management, 576–9
    antenatal management, 576
    failure of treatment, 579
    newborn, 577, 579, 713–14
    strategies, 577–9
    therapeutic procedures, 576–7
  pathogenesis, 569
  presentation, 573–4
    intracranial hemorrhage, 574
    predictors of disease, 574
  screening programs, 575–6
alloimmunization, 571–3, 715–17
  human leukocyte antigens (HLA), 715–16
  refractory patient management, 716–17
alpha 2A adrenoceptors, 898–9, 901
alpha-granules, 28, 60–1, 106–9, 655

contents, 107, 357, 403, 655, 993
defects, 108–9, 661–2
alpha/delta-storage pool deficiency, 662–3
Jacobsen/Paris–Trousseau syndrome, 522, 566, 662
Quebec platelet disorder, 109, 522, 662, 676–7
see also grey platelet syndrome
functions, 108, 655
means of study, 108
membrane, 107–8
origin, 108
properties, 106
secretion, 357, 358–60
Alport syndrome, 518–19, 565
alternagin, 165
aminomethyltrimethylpsoralen (AMT), 810–11
5-aminosalicylates, 911
anagrelide
essential thrombocythemia treatment, 632
side effects, 632
anemia
Fanconi anemia, 533
iron deficiency, 478
thrombocytosis and, 625
megaloblastic anemia, 533–4
pernicious anemia, 534
role in uremia, 839–40
see also aplastic anemia
angina
as outcome in clinical trials, 1007–8
stable angina, 734, 922
see also unstable angina
angiogenesis, 829, 876
annexin V, 383
antibiotics, 690
uremia and, 840
anticoagulants, 474
group IIA phospholipase A_2 and, 230–1
platelet aggregation and, 340–2, 944
antidepressants, 895
serotonin reuptake and, 897
antiendothelial cell antibodies, 617–18
antioxidants, 478
atherosclerotic lesion stabilization, 746
antiphospholipid antibodies, 551
antiplatelet antibodies, 694
assays, 546–7, 575
thrombotic thrombocytopenic purpura and, 617–18
antiplatelet therapy, 1013
acute coronary syndromes
non-ST elevation, 1017–19
ST elevation, 1019–24
agents which elevate cAMP, 473
atherothrombosis prevention, 1040–1
clinical trials, 753–4, 923–4, 970–2
see also clinical trial design
glomerular diseases, 844
GPIb receptor antagonists, 473
GPIIb–IIIa receptor antagonists, 473
history of, 964–5
inflammatory bowel disease, 911–13
influencing factors, 478–9
depression, 479
diet, 478
exercise, 478
genetic factors, 479
hormonal effects, 478
smoking, 478
inhibitors of platelet agonist receptors, 473
metastasis inhibition, 830–1
monitoring, 471–9, 945
agonists, 471–2
antiplatelet agents, 472–3
cAMP and cGMP levels, 476–7
drug plasma level assays, 477
flow cytometry, 475–6
platelet adhesion, 477
platelet aggregation, 473–4
platelet count, 474
platelet function analyser (PFA-100), 474
platelets and coagulation, 477–8
rapid platelet function analyser, 474–5
template bleeding time, 477
thromboxane B_2, 476
whole blood aggregometry, 475
percutaneous coronary intervention, 1024–32
peripheral vascular disease, 1054–9
arterial surgery and, 1056–7
future prospects, 1059
limb ischemic symptoms and, 1055–6
percutaneous endovascular revascularization and, 1057–9
prevention of vascular events, 1054–5
platelet signalling inhibitors, 473
populations as targets, 965–6
primary prevention, 1013
risks, 1042
secondary prevention, 1014–17, 1041–9
stroke, 1041–9
see also specific drugs
Antiplatelet Trialists Collaboration, 753–4
antisense oligodeoxynucleotides, 984
antisense oligonucleotides, 984
antithrombins, 349, 438, 958, 1040
antithrombotic gene therapy *see* gene therapy
anxiety disorders, 901
aplastic anemia, 529, 530–2
causes
alcohol, 531
chemicals, 530–1
drugs, 530
immune mechanism, 530

Cambridge Books Online © Cambridge University Press, 2009

aplastic anemia (*cont.*)
causes (*cont.*)
radiation, 531
viral infection, 531
idiopathic, 530
treatment, 532
apolipoprotein E (apoE), 443
gene disruption, 443
apoptosis
degradation of apoptotic cells, 231
hemolytic uremic syndrome and, 618–19
myelodysplastic syndrome and, 535, 536
promotion of by gene transfer, 983
thrombotic thrombocytopenic purpura and, 618–19
aprotinin, 694, 994
arachidonic acid, 9, 140, 221, 874
antiplatelet therapy monitoring, 472
metabolism, 221–3, 369, 432
in platelet–leukocyte interaction, 416
phospholipase C activation, 246
platelet aggregation and, 344
release of, 221, 369, 371
impaired release from membrane phospholipids, 663, 681
role of phospholipase A_2, 225, 228–9, 372
Arf GTPase subfamily, 206, 207
phospholipase D regulation, 250–1
argatroban
heparin-induced thrombocytopenia therapy, 598, 599
pharmacology, 598
arginine vasopressin (AVP)
platelet aggregation and, 346
see also desmopressin
Arp2/3 complex, 98
arterial eversion graft model, 507–8
advantages and disadvantages, 508
general applicability, 508
observations, 508
aspirin, 12, 473, 919–26, 964, 965–6, 995–6
antiplatelet therapy, 221, 422, 1066
acute ischemic stroke, 1041, 1042–4, 1046–9
benefits, 745, 1044
clinical trials, 753–4, 970–2
costs, 1043
dosages, 1043–4
monitoring, 476, 479
non-ST elevation acute coronary syndromes, 1017
percutaneous coronary intervention, 1024
peripheral vascular disease, 1054, 1055, 1056–9
primary prevention, 923–4, 1013
safety, 926, 1043
secondary prevention, 1014, 1042–4, 1046–9
ST elevation acute coronary syndromes, 1019–22
antithrombotic effects, 922
aspirin-sensitive asthma, 861
cyclooxygenase interaction, 12, 372, 435, 919, 920
aspirin resistance and, 921
effects on platelet function, 690
bacterial infection and, 791
inhibition of aggregation, 8, 12, 65, 341, 344
essential thrombocythemia therapy during pregnancy, 632
genetic variability in response, 970–2
glomerular disease treatment, 844
inflammatory bowel disease and, 911
mechanism of action, 919
pharmacodynamics, 920–1
aspirin resistance, 921
effects not related to thromboxane A_2, 920
effects on prostacyclin, 920
effects on thromboxane A_2, 920
pharmacokinetics, 919–20
side effects, 924–6, 1043
uremia and, 840
see also antiplatelet therapy
aspirin resistance, 921
aspirin-like defect, 663
asthma, 855
platelet involvement, 852–4, 855, 995
airway remodelling, 858
aspirin and NSAID sensitive asthma, 861
leukocyte adherence to endothelium, 859–61
platelet-derived mediators, 856–8
atherosclerosis, 4, 5, 329–30, 738–47, 948, 978, 1039
clinical consequences, 734–5
endothelial dysfunction and, 438, 439–40, 441, 443
future developments, 747
group IIA phospholipase A_2 role, 231
pathogenesis, 738–44
acute vascular events, 742–3
fatty streak, 741–2
fibroatheroma, 742
incorporation of mural thrombi, 744
inflammation role, 738–41, 1039
leukocyte recruitment, 739–41
unstable lesions, 742, 743, 1039
plaque characteristics, 727, 1039
calcification, 742
heterogeneity, 732
thrombogenicity, 732–3
plaque rupture, 742, 1039–40, 1066
active disruption, 727–8
passive disruption, 727
sequelae of, 1039–40
triggers of, 1039
progression, 727
restenosis, 744, 1056
stabilization of lesions, 746–7
antioxidant therapy, 746
high density lipoprotein (HDL), 746–7
lipid lowering, 746
vein grafts, 985
see also atherothrombosis

Cambridge Books Online © Cambridge University Press, 2009

atherothrombosis, 1039–40, 1053
platelet role, 744–5, 1039–40
preventive antiplatelet therapy, 1040–1
risk factors, 745–6
ATP, 1064
release of, 358
atrial fibrillation, 1049
atrial naturetic factor (ANF), 293
attention deficit disorder (ADD), 679
autism, 900
autoimmune thrombocytopenia, 537, 545–51
childhood
acute autoimmune thrombocytopenic purpura, 557, 558–61
chronic autoimmune thrombocytopenic purpura, 558, 560–1
therapy, 559–61
drug-induced, 584
see also idiopathic thrombocytopenic purpura

bacterial infections, 783–96
disseminated intravascular coagulation (DIC), 785–6
Ehrlichial infections, 783–4
infective endocarditis, 784–5
mechanisms of bacterial–platelet interactions, 787–90
anti-infective platelet responses, 789–90
bacterial ligands, 787–8
platelet receptors, 788–9
Rickettsial infections, 783–4
see also specific bacteria and diseases
Bartter syndrome, 695
batroxostatin, 831
benzene toxicity, 530–1
Bernard–Soulier syndrome (BSS), 5, 179, 520–2, 565, 639–42
deficiency, 9–10, 86, 182, 310–11, 313, 522, 639
diagnosis, 342, 492, 640
frequency, 683
genetic basis, 182, 520, 522, 639, 640–2, 762
platelet characteristics, 94, 329, 520–1, 522, 639–40
symptoms, 639
β-thromboglobulin (βTG), 107, 108, 404
as laboratory marker for thrombosis, 755, 756
inflammatory bowel disease and, 909–10
malignancy and, 827
measurement, 464, 755
bias, in clinical trials *see* clinical trial design
bipolar disorder, 900–1
bivalirudin, 958
bleeding time test, 460–1
anemia and, 839–40
antiplatelet therapy monitoring, 477
drug effects, 690–2
liver disease and, 695
blood banks, quality control of stored platelet concentrates, 491–2
bone marrow
disorders affecting platelet production, 529–37
acquired amegakaryocytic thrombocytopenic purpura (AATP), 532
aplastic anemia, 530–2
congenital amegakaryocytic thrombocytopenia (CAMT), 523, 537, 566
Fanconi anemia (FA), 533
infiltration by malignancy, 533
megaloblastic anemia, 533–4
myelodysplastic syndrome (MDS), 534–6
paroxysmal nocturnal hemoglobinuria (PNH), 532–3
platelet transfusion and, 711
regulation disorders, 536–7
TAR-syndrome (thrombocytopenia with absent radius), 523–4, 537, 566
thrombopoietin deficiency, 536–7
platelet production, 72, 528
transplantation, 532, 683
Borrelia burgdorferi, 788–9
botrocetin
agglutination induction, 348
antiplatelet therapy monitoring, 472
bovine diarrhea virus (BVDV), 808
bradykinin, 717
Brugia malayi, 816
Bruton's tyrosine kinase (Btk), 285
busulphan, essential thrombocythemia treatment, 631

C3b, 818
C-reactive protein (CRP), 817, 920, 965–6
caffeine, 478
calcium, 14
ADP effects, 128–9, 930
cyclic AMP effects, 297
entry into platelets, 260–7, 358
actin cytoskeleton relationship, 263–5, 266
regulation, 266–7
second messenger-evoked entry, 262–3
store-mediated entry, 263–6
via the P2X1 purinoceptor, 261–2
IP_3 and, 239, 260, 262
mobilization defects, 679–80
phospholipase A_2 regulation, 225
platelet aggregation and, 896
prostaglandin D_2 receptor effects, 152
release of stored calcium, 260
removal from cytosol, 267–8
role in platelet shape change, 98, 99–100, 324
uremia and, 838
calcium pump, 65
calpain, 245–6, 397
thrombotic thrombocytopenic purpura and, 611
calreticulin, 165
cancer *see* malignancy
capillary endothelium, 4
CapZ, 93, 95, 97
cardiolipin, 551
cardiopulmonary bypass, 694–5
platelet transfusion and, 713

Cambridge Books Online © Cambridge University Press, 2009

cathepsin G, 994
cleavage of platelet surface glycoproteins, 414, 994
platelet aggregation induction, 346–7, 413–14
platelet receptor activation, 413–14
cC1q-receptor, 163
CD9, 165
CD11b/CD18, 416–17, 875
CD23
FcϵRI interactions, 820–1
role in parasite elimination, 819–20
CD26, 789
CD31 (PECAM-1), 165, 405, 739
bacterial interaction, 789
CD36, 86–7, 162–3, 168
bacterial interaction, 789
deficiency, 87, 648, 678
functions, 87
structure, 86–7
CD40, 231
atherosclerosis and, 740, 948
CD40 ligand (CD40L), 405, 948, 949
CD47 (integrin-associated protein), 164, 210
cell adhesion
inflammation and, 393–5, 739–41
leukocyte–endothelium adhesive interaction, 739–41, 744
allergic diseases and, 859–61
platelet–leukocyte adhesive interactions, 416–19
see also platelet adhesion
cell cycle inhibition, 983
cell migration, 393–6
platelet migratory ability, 397–8
animal studies, 400–2
vascular smooth muscle cells, 978, 980
inhibition of, 984
see also chemotaxis
chaperonin-10, 888–9
Chediak–Higashi syndrome (CHS), 61, 106, 660
animal models, 660
molecular aspects, 660
chemokines, 395–6, 397–8, 403–5
receptors, 395–6, 397–8
chemotaxis, 393–405
platelet chemotactic effects on other cells, 403–5, 415
platelet chemotaxis in vitro, 398–400
platelet chemotaxis in vivo, 400–2
human studies, 402
platelet migratory ability, 397–8
animal studies, 400–2
childhood thrombocytopenia, 545, 556–66
acquired thrombocytopenia, 558–64
acute autoimmune thrombocytopenic purpura, 557, 558–61
chronic autoimmune thrombocytopenic purpura, 558, 560–1
heparin-induced, 564, 599
increased platelet consumption, 563–4
infections, 562–3
therapy, 559–61
see also alloimmune thrombocytopenia
diagnosis, 556–7
inherited thrombocytopenias, 564–6
inherited giant platelet disorders, 565–6
normal platelet volume, 566
small platelet size, 564–5
Chlamydia pneumoniae, 791
chronic autoimmune thrombocytopenic purpura (AITP) in childhood, 558
therapy, 560–1
chronic lymphocytic leukemia (CLL), 551
cilostazol, 691, 959, 1056
clinical trial design, 1003–11
active vs. control randomized trials, 1006–7
adherence to randomized treatments, 1007
bias minimization, 1004–5
proper randomization, 1004
therapeutic decisions should be based on the totality of information, 1005
unbiased analysis of results, 1004–5
unbiased ascertainment of outcomes, 1004
effect of cardiac interventions, 1009
importance of competing risks, 1011
minimizing random error and ensuring study power, 1006
moderate treatment benefits, 1005–6
the question, 1003
treatment benefit will be greatest during the treatment period, 1008–9
trials comparing various active treatments, 1006–7
unanticipated qualitative treatment effects are rare, 1008
use of composite outcomes, 1007–8
clopidogrel, 131–3, 135, 343, 690–1, 929, 1066
antiplatelet effects of, 933
antiplatelet therapy monitoring, 479
cost, 1046
inflammatory bowel disease therapy, 912
non-ST elevation acute coronary syndrome therapy, 1017
percutaneous coronary intervention and, 1025
peripheral vascular disease therapy, 1054
pharmacogenetic approach, 972–3
pharmacology, 930–4
clinical pharmacology, 934
metabolism and pharmacokinetics, 930–2, 972
mode of action on platelets, 932, 972
secondary prevention, 1014–15
stroke, 1044–6, 1049
side effects, 972–3, 1046
clot retraction, 5, 193–4, 381
measurement, 478
clot signature analyser (CSA), 461–2, 478
clotting factors *see specific factors*
clotting process *see* coagulation; thrombus formation
coagulant phospholipids (CPL), 382–3, 387
coagulation, 3–4, 460, 991–2, 1040
antiplatelet therapy monitoring, 477–8

Cambridge Books Online © Cambridge University Press, 2009

GPIIb–IIIa antagonist effects, 947–8
hypercoagulable-dependent thrombosis, 734
inflammation relationships, 991–2
platelet procoagulant activity, 381, 383–7
measurement, 466
platelet–monocyte interactions and, 415–16
see also platelet aggregation; thrombus formation
cofilin, 328
collagen, 8, 158–60, 165–6, 308–9
antiplatelet therapy monitoring, 472
phospholipase C activation, 240, 246–8
platelet interaction, 99–100, 113, 158, 766
adhesive interactions, 87–8, 158, 308–9
aggregation induction, 343–4, 462
cyclic AMP effects, 298
hemostasis and, 160
models, 168–9
thrombosis and, 160
von Willebrand factor interaction, 307–8
collagen receptors, 158, 160–3, 343, 766
accessory molecules, 163, 164–5
calreticulin, 165
CD9, 165
Fc receptors, 164
integrin-associated protein (CD47), 164
PECAM-1 (CD31), 165
protein disulphide isomerase, 164
CD36, 86–7, 162–3, 168
defects, 87, 648, 677–8
glycoprotein VI, 87–8, 99–100, 162, 166–9
integrin $\alpha 2\beta 1$, 160–2, 168–9
affinity regulation, 163–4
other receptors, 163
signalling, 165–8, 283–4, 298
second messenger pathways, 166
colon carcinoma, 828–9
therapy, 830
Cone and Platelet Analyser (CPA), 945
congenital amegakaryocytic thrombocytopenia (CAMT), 523, 537, 566
convulxin, 165, 769–70
copper coil model of arterial thrombosis, 503–4
advantages and disadvantages, 504
general applicability, 503–4
observations, 503
coronary arteries
occlusion, 743
stenosis, 742–3
unstable lesions, 742
cortactin, 325
corticosteroid treatment
alloimmune thrombocytopenia, 577, 579
idiopathic thrombocytopenic purpura, 547, 548
inflammatory bowel disease, 907–8
Crohn's disease, 907
clinical features, 907
etiopathogenesis, 908
multifocal mesenteric microinfarction, 908, 911
see also inflammatory bowel disease
cryoprecipitate, uremia and, 693–4
CXCR4 chemokine receptor, 29
cyclic AMP (cAMP), 13, 65, 114, 290, 433
antiplatelet therapy monitoring, 476–7
calcium uptake and, 297
collagen-stimulated platelet activation and, 298
elevation of, 473, 691
G protein interaction, 206, 207–10
inhibition of platelet activation, 296, 732
IP_3 receptor regulation, 297–8
prostacyclin receptor interaction, 149
prostaglandin D_2 receptor interaction, 152
protein phosphorylation and, 296–7
regulation by phosphodiesterases, 295–6, 958–9
signal transduction and, 297
uremia and, 838
see also adenylyl cyclase; phosphodiesterases
cyclic AMP-dependent protein kinase *see* protein kinase A
cyclic flow variants (CFVs), 442, 500–2
cyclic GMP (cGMP), 13–14, 290, 295–6, 434
antiplatelet therapy monitoring, 476–7
inhibition of platelet activation, 298
protein phosphorylation and, 298
regulation, 958–9
see also phosphodiesterases
cyclic GMP-dependent protein kinase (PKG), 148, 294
cyclic thrombocytopenia, 537
cyclooxygenases (COX), 12, 140, 222, 369–70, 981
aspirin interaction, 12, 372, 435, 919, 920
aspirin resistance and, 921
defects, 663, 681–2
gene transfer to sites of arterial injury, 981–2
inhibitors
anti-inflammatory effects, 995–6
reversible COX-1 inhibitors, 955
see also specific drugs
isoforms, 369–70
thromboxane A_2 synthesis and, 372, 374, 375, 376–7, 435
CYP2CP gene polymorphism, 967–8, 972
cystathione β-synthase (CBS) gene, 443
cysteinyl leukotrienes, 858
cytochalasins, 46
cytokines, 396, 991–3
hemolytic uremic syndrome and, 618
role in febrile reactions to platelet transfusion, 717
thrombotic thrombocytopenic purpura and, 618
see also chemokines; *specific cytokines*
cytomegalovirus (CMV)
megakaryocyte interactions, 808
transmission by platelet transfusion, 718, 809
cytoskeleton, 14, 93–8
activated platelet, 95–8
actin assembly regulation, 96–8

Cambridge Books Online © Cambridge University Press, 2009

cytoskeleton (*cont.*)
activated platelet (*cont.*)
actin nucleation by WASp and Arp2/3 complex, 98
assembly defects, 682
migratory ability and, 397
phospholipase C and, 248–9
resting platelet, 93–5
signalling leading to platelet shape change, 98–100
cytosolic calcium increase, 99–100
new production of membrane polyphosphoinositides, 100
small GTPases, 100
see also actin filaments; microtubules

danaparoid
heparin-induced thrombocytopenia therapy, 598–9, 600
children, 599
pregnancy, 599
pharmacology, 598
deep vein thrombosis (DVT), 753, 757
aspirin therapy, 753–4
laboratory markers, 755–7
beta thromboglobulin, 755
cross-sectional studies, 755–6
platelet factor-4, 755
predictive value, 756–7
soluble P selectin, 755
platelet imaging, 757
see also peripheral vascular disease; thrombosis
demarcation membrane system, 27
platelet production from, 72
dendritic platelets, 45–6, 47
dense bodies/granules, 28, 59, 60, 104–6, 655
contents, 104–5, 357, 403, 655
defects, 106, 657–61, 676
Chediak–Higashi syndrome, 61, 106, 660
hereditary thrombocytopenias, 661
Hermansky–Pudlak syndrome, 60, 65, 106, 659–60
see also storage pool disease
functions, 105–6, 358, 359–60, 655
means of study, 105
membrane, 105, 359
origin, 105
properties, 104, 358
secretion, 357, 358
protein kinase C-independent secretion, 361–2
uremia and, 838
dense tubular system (DTS), 41–2, 63–5
depression, 479, 895, 896
role of platelet adrenoceptors, 898–9
serotonin reuptake and, 896–7
Dermatophagoides pteronyssinus allergy, 854
desmopressin (DDAVP), 346, 666, 683
uremia and, 693
diabetes mellitus, 313, 440, 745
endothelial dysfunction and, 441–2
diacylglycerol (DAG), 14, 238, 239, 731
cyclic AMP effect, 297
production of, 249, 253
receptor, 272
dialysis, 599
uremia and, 693, 839
complications, 840–1
diapedesis *see* cell migration; chemotaxis
dietary influences on platelet function, 478, 1065–6
see also fish oil supplementation
diethylcarbamazine (DEC), 819
DiGeorge syndrome, 520, 565
Dipetalonema viteae, 816
dipyridamole, 422, 473, 691, 959, 964, 1064
cost, 1048
glomerular disease treatment, 844
side effects, 1048
stroke secondary prevention, 1046–8, 1049
disintegrins, 831
disodium cromoglycate, 995
disseminated intravascular coagulation (DIC), 347, 536, 695, 785–6, 992
childhood, 563
thrombocytopenia and, 786
Döhle bodies, 518–19, 565
Down's syndrome, 682
drug effects on platelet function, 689–92
antibiotics, 690
anticoagulants, 691–2
aspirin, 690
cyclic nucleotide levels, 691
fibrinolytic agents, 691–2
GPIIb–IIIa receptor antagonists, 691
non-steroidal anti-inflammatory drugs (NSAIDs), 690
thienopyridines, 690–1
see also drug-induced thrombocytopenia; *specific drugs*
drug-induced thrombocytopenia, 473–4, 530, 552–3, 584–600
clinical presentation, 585, 587–8
diagnosis, 585, 587
drugs responsible, 585, 586–7
gold, 553, 584
GPIIb–IIIa receptor antagonists, 587–91
heparin *see* heparin-induced thrombocytopenia (HIT)
quinidine, 553
pathogenesis, 584–5, 590–1
treatment, 585, 588–9
dynein, 95
dyserythropoiesis, 517
dysproteinemias, 696

E-selectin, 393, 394
early pregnancy factor (EPF), 888
ecto-ADPase, 437, 440–1
endothelial dysfunction and, 441
EDTA, 340

Cambridge Books Online © Cambridge University Press, 2009

Ehlers–Danlos syndrome, 682
Ehrlichial infections, 783–4
eicosapentaenoic acid, 222, 1065–6
embryo-derived platelet-activating factor, 885–6
  autocrine actions of, 890
  physiological actions of, 888
emperipolesis, 25
endomitosis, 27
endoplasmic reticulum, 28
endothelial cell growth factor (ECGF), 107, 826
endothelial dysfunction, 432, 437–43
  animal models, 442–3
  associated conditions, 441–2
    atherosclerosis, 438, 439–40, 441, 443, 738–9
    diabetes mellitus, 441–2
    hypercholesterolemia, 438, 441, 443
    hyperhomocysteinemia, 442, 443
    hypertension, 441
  exposure to subendothelium, 437
  increased platelet activation, 439–41, 746, 869
    ecto-ADPase, 440–1
    nitric oxide, 439–40
    prostacyclin, 439
  plasminogen activator inhibitor and, 438
  thrombin and, 438–9
  tissue factor and, 439
  von Willebrand factor and, 437–8
endothelial protein C receptor (EPCR), 991
endothelin, 434–5
endothelin receptors, 435
endothelium, 4, 432, 738, 991, 1038–9
  antiendothelial cell antibodies, 617–18
  endothelial cell apoptosis, 618
  leukocyte adhesion, 739–41, 744, 992–3
    allergic diseases and, 859–61
  molecular determinants of function, 432–7
    antiplatelet substances, 435–7
    vasoactive substances, 432–5
  platelet–endothelial interaction, 744, 869
    during inflammation, 184, 739–40, 871, 992
    see also platelet adhesion
  see also endothelial dysfunction
endothelium-derived hyperpolarizing factor (EDHF), 434, 738
endothelium-derived relaxing factor (EDRF) *see* nitric oxide
Enterococcus faecalis, 785
eosinophilic fasciitis, 532
eotaxins, 395, 398
epidermal growth factor (EGF), 107, 826
epinephrine (adrenaline), 9, 210, 324
  antiplatelet therapy monitoring, 472
  calcium entry regulation, 267
  platelet aggregation and, 345
    impaired responsiveness, 678–9
epinephrine receptor, 293
Epstein syndrome, 518, 565, 682
Epstein–Barr virus, aplastic anemia and, 531
eptifibatide, 589, 691, 943–4
  acute coronary syndrome therapy
    non-ST elevation, 1019
    ST elevation, 1022–4
  percutaneous coronary intervention and, 1031–2
  see also GPIIb–IIIa antagonists
eristostatin, 831
erythrocytes *see* red blood cells
erythropoietin (EPO), 287
  anemia correction, 840
  human recombinant erythropoietin (rhEPO), 840
Escherichia coli, 786, 788
essential thrombocythemia (ET), 623, 625–33
  bleeding in, 628
  children, 633
  diagnosis, 625–8
    differential diagnosis, 627
    platelet abnormalities, 626–7
    platelet count, 626
  disease transformation, 629
  pregnancy and, 632–3
  surgery and, 633
  thrombosis and, 628–9
  treatment, 630–3
    anagrelide, 632
    busulphan, 631
    hydroxyurea (HU), 630–1
    interferon, 631–2
    lower-risk patients, 630
estrogen, 478
  uremia and, 693
ethanol, 293
ethnic specificity, 967
Ets transcription factors, 761, 764, 771
exercise, 478
exocytosis, 357–8
  cytoskeletal fibres and, 362
  GTP-binding proteins in, 364–5
    presence on secretory vesicles, 364
    RabGTPase family, 364–5
  plasma membrane fusion, 362
    SNARE hypothesis, 362–4
  protein kinase C role, 360–2
exosomes, 360

F2-isoprostanes, 745
factor IX, 384, 993, 1040
factor IXa, 382, 1040
  receptor, 386
factor V, 358, 383–4, 439, 993, 1040
factor V Quebec syndrome, 109, 522, 662, 676–7
factor Va (FVa), 230, 382, 383, 991, 1040
  receptor, 386
factor VII, 439

Cambridge Books Online © Cambridge University Press, 2009

factor VIIa, 439
factor VIII, 382–3, 384, 439, 993, 1040
factor VIIIa, 1040
  receptor, 386
factor X, 384, 439, 991, 993, 1040
factor Xa (FXa), 230–1, 382, 439, 992, 993, 1040
  receptor, 386
factor XIa, 386
  receptor, 386
familial platelet disorder with predisposition to acute myelogenous leukemia (FPD/AML), 522
Fanconi anemia (FA), 533
fatty streak, 741–2
Fc receptors, 164, 167
  bacterial interaction, 789
  FcεRI role in parasite elimination, 819–20
    CD23 regulatory role, 820–1
  heparin-induced thrombocytopenia and, 592–3
febrile reactions to platelet transfusion, 717–18
Fechtner syndrome, 519, 565
fetal blood sampling (FBS), 575–6, 578–9
  frequency, 579
fibrin, 3–4, 195, 384–5, 1040
  platelet adhesion, 310
  platelet procoagulant activity stimulation, 387–9
  soluble fibrin monomer (sFM), 785
fibrinogen, 10, 183, 195, 309–10, 731, 1063
  afibrinogenemia, 683
  integrin interaction, 82, 286
    see also GPIIb–IIIa complex
  malignancy and, 827
  platelet adhesion and, 309–10
  role in inflammation, 948
  role in platelet aggregation, 188, 192, 286, 459–60, 941–2
fibrinogen receptors, 188–95
  integrin $\alpha 5\beta 1$, 194
  integrin $\alpha v\beta 3$, 194–5
  mobility, 48, 53
  see also GPIIb–IIIa complex
fibrinolysis, GPIIb–IIIa antagonist effects, 948
fibroatheroma, 742
fibromyalgia, 901–2
fibronectin, 309
fibronectin receptor, 311–12
fibroproliferative lesion prevention by gene therapy, 982–5
  antisense oligodeoxynucleotides and decoy sequences, 984
  antisense oligonucleotides, 984
  apoptosis promotion, 983
  cell cycle inhibition, 983
  inhibition of vascular smooth muscle cell migration, 984
  nitric oxide synthase promotion, 984–5
  vascular endothelial growth factor promotion, 984–5
fibrosarcoma, 825
filamin A, 94
  GPIb–IX–V interaction, 184
filarial infections, 816
filopodia formation, 326–7, 329
fish oil supplementation, 222, 844, 1065–6
  glomerular disease therapy, 844
fixation, flow cytometry, 489
flightless-I, 97
flow cytometry, 465–6, 471, 485–93
  antiplatelet therapy monitoring, 475–6, 491
    receptor occupancy assay, 475–6
  clinical applications, 491–3
    circulating activated platelet detection, 465, 491
    circulating platelet aggregate detection, 466
    leukocyte–platelet aggregates, 491
    platelet-derived microvesicles/microparticles, 465–6, 492–3
    quality control of stored platelet concentrates, 491–2
    reticulated platelets, 492
    storage pool disease diagnosis, 492
    surface glycoprotein deficiency diagnosis, 492
  methodological issues, 487–91
    advantages of flow cytometry, 487–8
    blood drawing, 488
    calibration of the flow cytometer, 490
    choice of monoclonal antibodies, 489–90
    detection of activation-dependent decrease in GPIb-IX-V complex, 490–1
    disadvantages of flow cytometry, 488
    expression of antibody binding, 490
    fixation, 489
    minimizing platelet aggregates, 488–9
  monoclonal antibodies, 486–7, 489–90
foam cells, 741–2
focal adhesion kinase (FAK), 283, 284–5, 286, 287
FOG cofactor, 517, 518, 528, 770
folic acid deficiency, 478, 533–4
Folts coronary thrombosis model, 442, 499–502
  advantages and disadvantages, 502
  general applicability, 502
  observations, 501–2
n-formyl-methionyl-leucyl-phenylalanine (fMLP), 871–2, 873–4
Friend's leukemia integration-1 (Fli-1), 761, 764
frozen storage, 710

G proteins, 14, 99, 114, 204–6, 208, 732
  activation defects, 679
  as integrators of signal transduction, 207
  $\beta\gamma$ subunit role, 212, 244
  G_{12}, 206, 211, 325
  G_{13}, 211, 325
  G_{15}/G_{16}, 211
  G_h, 211, 248
  G_i (inhibitory G-protein of adenylyl cyclase), 206, 209–10
  G_p, 240
  G_q, 206, 210–11, 286, 325, 930
  G_s (stimulatory G-protein of adenylyl cyclase), 206, 207–9
  G_z, 211

Cambridge Books Online © Cambridge University Press, 2009

phospholipase C activation, 239–40, 243–4, 286
phospholipase D regulation, 250–1
phosphorylation, 274
platelet shape change and, 325
receptor interactions
prostacyclin receptor, 149
thromboxane A_2 receptor, 145–6
GATA transcription factors, 761, 765, 771
GATA-1, 517–18, 528, 761, 770–1
GPIb gene regulation, 762–3
GATA-2, 761
GATA-3, 761
gC1qR platelet receptor, 788
gelsolin, 95, 96–7, 249
role in platelet shape change, 327
gene therapy, 978–86
antithrombotic gene therapy, 978–82
cyclooxygenase-1 gene transfer to sites of arterial injury, 981–2
gene restoration of tissue factor pathway inhibitor, 982
gene restoration of tissue plasminogen activator, 981
gene transfer techniques, 980
thrombomodulin gene transfer, 980
fibroproliferative lesion prevention, 982–5
antisense oligodeoxynucleotides and decoy sequences, 984
antisense oligonucleotides, 984
apoptosis promotion, 983
cell cycle inhibition, 983
inhibition of vascular smooth muscle cell migration, 984
nitric oxide synthase promotion, 984–5
vascular endothelial growth factor promotion, 984–5
vein grafts and, 985–6
implications for gene delivery, 985
prevention of vein graft deterioration, 985–6
see also pharmacogenetics
genistein, 422
Glanzmann thrombasthenia (GT), 643–6
defect, 83–4, 188, 189, 286–7, 313
diagnosis, 341, 492
genetics, 195, 643–6
glomerular diseases, 842–4, 876
antiplatelet agent effects on, 844
role of platelet secretory products, 842–3
role of platelet-derived growth factors, 843–4
glucose-6-phosphatase deficiency, 682
glutathione, 861
glutathione peroxidase, 443, 861
glycocalicin, 465
glycocalyx, 41, 43
glycogen storage disease type-1, 682
glycoproteins (GP), 43, 80–1, 107, 730–1
cleavage by cathepsin G, 414
GPI, 9–10
GPIb, 731, 762–4
bacterial interactions, 789
gene regulation, 762–4
GPIbα, 762–3
GPIbβ, 763–4
mutations, 642, 763–4
polymorphisms, 643, 765–6
receptor antagonists, 473
signal transduction through, 765
uremia and, 838
GPIb–IX–V receptor complex, 51–4, 81, 84, 179–85
activation-dependent decrease in surface expression, 490–1
cytoplasmic interactions, 184
deficiency *see* Bernard–Soulier syndrome
function, 85–6, 121–2, 183–4, 304
gene structure and expression, 181–2
megakaryocytopoiesis and, 184
platelet adhesion and, 310–11, 459
platelet–endothelial interaction and, 184
polymorphisms, 181
structure, 84–5, 179–81
thrombin binding, 183–4
von Willebrand factor interaction, 85–6, 94, 179, 181, 183
GPIIb–IIIa complex *see* GPIIb–IIIa antagonists; GPIIb–IIIa complex
GPIV *see* CD36
GPIX, 764
gene regulation, 764
GPV, 764
gene regulation, 764–5
GPVI, 87–8, 99–100, 162, 166, 168–9, 246
bacterial interaction, 789
deficiency, 87–8, 647–8, 678
function, 87, 246, 248, 769
gene regulation, 769
platelet adhesion and, 311
polymorphic expression, 769–70
signalling, 166–8
structure, 87
see also integrins
gold-induced thrombocytopenia, 553, 584
see also drug-induced thrombocytopenia
GPIIb–IIIa antagonists, 473–8, 479, 940–50, 965
acute coronary syndrome therapy
non-ST elevation, 1017–19
ST elevation, 1022–4
coagulation activity, 947–8
effects on platelet function, 691, 944–5
agonist effect, 945
anticoagulant effect, 944
fibrinolysis and, 948
genetic variability in response, 969–70
induced thrombocytopenia, 587–91, 691
clinical presentation, 587–8
frequency, 587
human antichimeric antibodies (HACAs), 591
pathogenesis, 590–1

Cambridge Books Online © Cambridge University Press, 2009

GPIIb–IIIa antagonists (*cont.*)
induced thrombocytopenia (*cont.*)
pseudothrombocytopenia, 589–90
treatment, 588–9
inflammation and, 948
integrin crossreactivity, 945–6
LIBS epitope expression and, 946
monitoring, 475–8, 479, 491, 945
orally available GPIIb–IIIa antagonists, 948–9
percutaneous coronary intervention and, 1025–32
primary prevention, 1013
secondary prevention, 1015–17
stroke, 1048
see also abciximab; eptifibatide; tirofiban
GPIIb–IIIa complex (integrin αIIbβ3), 10, 47, 81–4, 188–95, 731, 770–3, 940
activation, 731
activation defects, 680–1
allelic variants, 771
bacterial interactions, 788–9
calcium entry regulation, 266–7
conformational changes, 475, 486, 731
deficiency *see* Glanzmann thrombasthenia
fibrinogen engagement, 192
fibrinogen recognition, 194, 731
function, 82–3, 179, 183, 188, 191–4, 286–7
gene regulation, 770–1
metastasis and, 827–8
monoclonal antibodies to, 831, 913
phospholipase C activation, 240
phospholipase D activation, 252
platelet adhesion and, 311
platelet aggregation and, 459–60, 940–3
inside-out signalling, 940–1
outside-in signalling, 941–2
platelet agonist receptors, 942–3
polymorphisms, 771–3, 969–70
postreceptor occupancy events, 192–4
prothrombin binding, 386
receptor activation, 191–2
receptor mobility, 47–54
structure, 81–2, 189–91
thrombosis and, 729–30
tumour cells, 826, 827–8
uremia and, 838–9
see also glycoproteins; GPIIb–IIIa antagonists
graft-vs.-host disease (GVHD), 532
platelet transfusion and, 718
granules, 10–11, 27–8, 59, 357
contents, 357
dysfunctions, 365
plasma membrane fusion, 362
SNARE hypothesis, 362–4
secretion, 357–65, 655–6
cytoskeletal fibres and, 362
GTP-binding proteins in, 364–5
protein kinase C role, 360–1
see also alpha-granules; dense bodies/granules; lysosomes
granulocyte–macrophage colony stimulating factor (GM–CSF), 71
granulophysin, 105
grey platelet syndrome (GPS), 59–60, 108–9, 214, 519–20, 566, 661–2
animal models, 661–2
clinical features, 661
laboratory findings, 661
GRK kinases, 117
GTPases *see* small GTPases
guanidinosuccinic acid (GSA), 839
guanine nucleotide exchange factors (GEF), 100, 364–5
guanylyl cyclase, 293

hairy-cell leukemia, 696
hantavirus, 808
hantavirus pulmonary syndrome (HPS), 808
headache, 900
heart disease
aspirin therapy benefits, 924
platelet shape change and, 329
platelet–leukocyte interaction and, 421
heat-shock proteins (Hsps), 327
Helicobacter pylori, 786, 791
Helinx Technology, 811
hemangioma, Kasabach–Meritt syndrome, 563
hemodialysis, 599
uremia and, 693, 839
complications, 840–1
hemolytic uremic syndrome (HUS), 329, 536, 610
apoptosis and, 618–19
epidemiology, 610
pathophysiologic mechanisms, 616
hemorrhage
alloimmune thrombocytopenia, 573–4
intracranial hemorrhage, 574, 576, 578
antiplatelet therapy risks, 1042
aspirin adverse effects, 924–6
essential thrombocythemia and, 628
platelet transfusion trigger, 712–13
uremia and, 837
see also hemostasis
hemorrhagic fever with renal syndrome (HFRS), 808
hemostasis, 3, 381–2, 459–60, 760, 1061–5
platelet–collagen interaction importance, 160
tests of
bleeding time, 460–1, 477
clot signature analyser (CSA), 461–2, 478
PFA-100 in vitro platelet function analyser, 461
von Willebrand factor role, 179, 304
hemostatic plug formation, 4
Hemostatus test, 477
heparin, 230, 340–1, 474, 958
anti-inflammatory effects, 995
effects on platelet function, 691

Cambridge Books Online © Cambridge University Press, 2009

inflammatory bowel disease therapy, 911–12
platelet–leukocyte interaction modulation, 421–2
heparin cofactor II, 349
heparin-induced thrombocytopenia (HIT), 347, 474, 493, 552–3, 564, 591–600
diagnosis, 595
frequency, 594–5
non-immune, 591
pathogenesis, 591–4
antibodies involved, 592
endothelial cells in HIT, 593
platelet receptors and, 592–3
polysaccharides involved, 592
proteins involved, 591–2
thrombin in, 593–4
platelet transfusion and, 715
re-exposure to heparin, 600
therapy, 595–600
adjunctive treatments, 599–600
argatroban, 598, 599
danaparoid, 598–9
hirudin, 596, 599
see also drug-induced thrombocytopenia
hepatic growth factor (hGF), 985
hepatitis
aplastic anemia and, 531
hepatitis C virus (HCV), 808
transmission by platelet transfusion, 718, 809
hepatocyte growth factor (HGF), 826
hereditary gelsolin-related amyloidosis, 329
Hermansky–Pudlak syndrome (HPS), 60, 65, 106, 659–60
animal models, 660
clinical features, 659
laboratory findings, 659–60
molecular aspects, 660
prevalence, 659
hexokinase deficiency, 682
high density lipoprotein (HDL), 746–7
hirudin, 340, 958
heparin-induced thrombocytopenia therapy, 596, 599
children, 599
pregnancy, 599
inflammatory disorders and, 995
pharmacology, 596
hirulog, 995
histamine, 405, 856, 995
headache and, 900
histamine releasing factors (HRF), 405
Hodgkin's disease, 551
homocystinuria, 442
hormone replacement therapy (HRT), 769
human antichimeric antibodies (HACAs), 591
human immunodeficiency virus (HIV), 807–8
idiopathic thrombocytopenic purpura and, 550
platelet interactions, 807–8
thrombocytopenia and, 531, 550
transmission by platelet transfusion, 718, 809
human leukocyte antigens (HLA), 570
alloimmunization, 571–3, 715–16
human platelet antigen (HPA), 570–1
alloimmunization, 571–3
antigen testing, 575
screening programs, 575–6
human T-cell lymphotrophic viruses (HTLV-I), 809
12-hydroxyeicosatetraenoic acid (12-HETE), 856–8, 873–4
12-hydroxyperoxyeicosatetraenoic acid (12-HPETE), 223, 861, 873–4
5-hydroxytryptamine (5-HT) *see* serotonin
hydroxyurea (HU)
essential thrombocythemia treatment, 630
leukemogenic potential, 630–1
hypercholesterolemia, 313, 440, 746
atherosclerosis and, 441
endothelial dysfunction and, 438, 441, 443
hyperhomocysteinemia, 478, 745
endothelial dysfunction and, 442, 443
hypersplenism, 536, 543–4
hypertension, 329, 440, 745, 898
aspirin and, 924, 925
endothelial dysfunction and, 441
hypertrophic osteoarthropathy, 869

ICAM-1, 394
ICAM-2, 394
idiopathic aplastic anemia, 530
idiopathic thrombocytopenic purpura (ITP), 32–3, 529, 544, 545–51, 694
HIV-associated ITP, 550
investigations, 545–6
platelet antibody testing, 546–7
pregnancy and, 549–50, 558
treatment, 547–9
childhood, 559–61
chronic refractory ITP, 548, 561
emergency treatment, 548, 561
experimental treatments, 549
platelet transfusion, 713
splenectomy, 548
see also autoimmune thrombocytopenia
IgA myeloma, 696
IgG multiple myeloma, 696
Iloprost, 422
imidazoline receptors, 899
imipramine, 898, 902
immune thrombocytopenic purpura *see* idiopathic thrombocytopenic purpura (ITP)
immunoglobulin E (IgE), 396, 398
interactive receptors for, 819–21
platelet stimulation against parasites, 816–17
role in allergic diseases, 854–5
immunoreceptor tyrosine-based activation motif (ITAM), 87, 99–100, 246, 283

Cambridge Books Online © Cambridge University Press, 2009

impedance platelet aggregometry, 339, 340
impulsivity, 900
indobufen, 955
infective endocarditis, 784–5
inflammation, 228–9, 393–4, 402
coagulation relationships, 991–2
drugs affecting platelet inflammatory function, 995–6
antihistaminergic drugs, 995
antiserotonergic drugs, 995
heparin, 995
hirudin/hirulog, 995
NSAIDs, 995–6
erythrocyte interactions, 873
GPIIb–IIIa antagonist effects, 948
leukocyte–endothelial interaction, 739
asthma and, 859–61
platelet involvement, 870–6, 992–4
antiphlogogenic functions, 876
endothelial interactions, 184, 739–40, 871, 992
inhibitory effects of inflammatory cells, 876
mechanisms of inflammatory cell interactions, 874–5
monocyte interactions, 872–3
neutrophil interactions, 871–2, 874, 875, 876
transcellular metabolism, 873–4
thrombocytosis and, 624, 869
vascular disease and, 419, 422–3
atherosclerosis, 738–41, 1039
see also cell migration; chemotaxis
inflammatory bowel disease (IBD), 907–13
clinical features, 907
etiopathogenesis, 908
platelets in, 908–11
explanations for abnormalities, 910
pathogenic implications, 910–11
platelet aggregation, 908–9
platelet number, 908
platelet size and survival, 908
serum proteins and platelet surface markers, 909–10
thromboembolic disease and, 907
treatment, 907–8
5-aminosalicylates, 911
antitumour necrosis factor-alpha (TNFα) antibodies, 912
aspirin and NSAIDs, 911
GPIIb–IIIa antibodies, 913
heparin, 911–12
therapeutic implications of platelet dysfunction, 911–13
thromboxane synthesis inhibitors, 911
ticlopidine and clopidogrel, 912–13
inflammatory disorders, 869–70
see also allergic diseases; asthma; inflammation; inflammatory bowel disease
inositol 1,3,4,5-tetrakisphosphate (IP4), 239
inositol 1,4,5-trisphosphate (IP_3), 14, 99, 238–9, 731
calcium and, 239, 260, 262
store-mediated calcium entry, 265–6, 433
IP_3 receptor, 297
cyclic AMP-dependent regulation, 297–8
phospholipase C activation and, 239, 244, 260
insulin-like growth factor (IGF), 107
Integrilin, 341
integrin-associated protein (CD47), 164, 210
integrins, 47, 81–4, 194–5, 394, 730
β1 (VLA) integrins, 394
integrin α2 gene, 766–7
alleles, 766–7
polymorphisms, 766, 767–8
integrin $\alpha 2\beta 1$, 160–2, 168–9, 248
affinity regulation, 163–4
deficiency, 646–7
function, 82–3
hormonal regulation of expression, 769
platelet adhesion and, 311
polymorphism, 312
signalling, 168
integrin $\alpha 5\beta 1$, 194, 946
integrin αIIbβ3 *see* GPIIb–IIIa complex
integrin $\alpha v\beta 3$, 194–5, 945–6
signalling, 168, 284
see also glycoproteins
interferon (IFN)
essential thrombocythemia treatment, 631–2
side effects, 631–2
interleukin 1 (IL-1), 396, 398, 405, 991, 992–3
malignancy and, 827
myelodysplastic syndrome and, 535
interleukin 3 (IL-3), 34
interleukin 6 (IL-6)
hemolytic uremic syndrome and, 618
platelet stimulation against parasites, 818
thrombopoietic activity, 34
thrombotic thrombocytopenic purpura and, 618
interleukin 11 (IL-11), 34
interleukin β, 107
intermittent claudication, 959, 1053–6
see also peripheral vascular disease
intimal hyperplasia, 419–20
intracranial hemorrhage (ICH)
alloimmune thrombocytopenia, 574, 576, 578
aspirin and, 926
intravenous immunoglobulin (IVIG), 577, 579
iron deficiency, 478
thrombocytosis and, 625
isoprostanes, 745

Jacobsen (Paris–Trousseau) syndrome, 522, 566, 662
JAK family tyrosine kinases, 285
jaracetin, 165
jararhagin, 165

Kasabach–Meritt syndrome, 563
ketanserin, 956

Cambridge Books Online © Cambridge University Press, 2009

ketotifen, 995
kidney disease *see* renal disease

L-selectin, 393, 394
Lactobacillus, 788
lamellipodia, 395
  formation, 326–7, 329, 395
lamifiban, 1019, 1022–4
laminin, 310
large heat shock proteins (Hsps), 327
LAT (linker for activation of T cells), 247
lepirudin, heparin-induced thrombocytopenia therapy, 596, 599
  children, 599
leucine-rich motif (LRM) family, 84–6
  deficiency, 86
  function, 85–6
  structure, 84–5
leukemia, 696
  chronic lymphocytic leukemia (CLL), 551
  hairy-cell leukemia, 696
  see also acute leukemia; chronic lymphocytic leukemia
leukocytes
  atherosclerosis and, 739–41
  endothelial interaction, 739–41, 744, 992–3
    allergic diseases and, 859–61
  migration, 393–6
  platelet interactions, 412–23, 740–1
    adhesive mechanisms, 416–19
    down-regulation of platelet function, 414
    leukocyte activation by platelets, 414–15
    leukocyte down-regulation by platelets, 415
    leukocyte–platelet aggregate detection, 491
    pharmacology of, 421–3
    platelet–monocyte interactions relevant to coagulation, 415–16
    role in thrombosis, 419
    role in vascular damage, 419–21
    transcellular arachidonic acid metabolism in, 416
    up-regulation of platelet function, 412–14
  see also monocytes; polymorphonuclear leukocytes (PMNs)
leukotrienes, 858, 873–4
ligand-attenuated binding site (LABS), 475
ligand-induced binding sites (LIBS), 82, 193, 475, 486
  GPIIb–IIIa antagonists and, 946
ligand-regulated binding sites (LRBS), 475
lipid lowering therapy, 746
lipopolysaccharides (LPS), 783, 788
lipoteichoic acid (LTA), 784, 787
lipoxygenase, 222, 856–8
liquid storage, 709–10
lithium, 1062
liver disease, 536–7, 695
Loa loa, 816
low density lipoprotein (LDL), 738, 746, 1039
  group IIA phospholipase A_2 role in hydrolysis, 231
  oxidized (ox-LDL), 440, 738, 739, 741–2, 746, 1039
lung carcinoma, 825, 828
  therapy, 830
lungs, platelet production site, 73–4
lymphocyte-associated function antigen-1 (LFA-1), 394
lysophosphatidic acid (LPA), 212, 214, 239, 328
lysosomal associated membrane proteins (LAMPs), 109
lysosomes, 28, 61, 109–10, 655
  contents, 109, 347, 405, 655
  defects, 110
  functions, 110, 655–6
  means of study, 110
  membrane, 109
  origin, 109
  properties, 109
  secretion, 357–8, 655
  structure, 109

macrophage antigen-1 (Mac-1), 394, 945–6
  platelet–leukocyte adhesive mechanisms, 416–17, 419
  vascular damage/disease and, 420, 421, 422
macrophage derived chemokine (MDC), 396, 398, 855
macrophage inflammatory proteins (MIPs), 395–6, 398, 404–5
macrothrombocytopenia, 313, 517–18
  autosomal dominant, 518–21
  autosomal recessive, 522
  X-linked recessive, 517–18
malaria, 815–16
malignancy
  bone marrow infiltration, 533
  platelet changes associated with, 824–6
  platelet–tumour cell interactions, 826–31
    angiogenesis, 829
    immune response to tumour, 829–30
    pharmacological implications, 830–1
    platelet-mediated tumour cell adhesion and metastasis, 827–9
    platelet-released products, 826–7
    tumour-cell induced platelet aggregation, 826
  thrombocytosis and, 624
  see also metastasis
mania, 895, 900–1
MARCKS (myristoylated alanine-rich C kinase substrate), 360–1
Marfan's syndrome, 682
matrix metalloproteinases (MMPs), 396–7, 398, 984
May–Hegglin syndrome, 312, 518, 519, 564, 682
  platelet shape change and, 329
mean platelet volume (MPV)
  essential thrombocythemia and, 626
  heterogeneity, 70, 71–2
  inflammatory bowel disease and, 908, 910
  myocardial infarction and, 74–5, 76
  unstable angina and, 75, 76
Mediterranean thrombocytopenia, 566, 784
megakaryocyte (MK), 5–6, 25, 528
  chemotaxis, 29

Cambridge Books Online © Cambridge University Press, 2009

megakaryocyte (MK) (*cont.*)
colony formation, 33
diploid precursor, 26
endomitosis, 27
fragmentation in bone marrow, 72
gene regulation, 760–1
hormonal regulation, 769
knockout mice models, 29–30
localization, 25–6
maturation and differentiation, 26–8, 33
alpha granule origin, 108
demarcation membrane system, 27
dense granule formation, 105
endoplasmic reticulum, 28
lysosome origin, 109
protein kinase C and, 277–8
regulation, 31–5
secretion granules, 27–8
see also megakaryopoiesis; thrombopoietin (TPO)
myocardial infarction and, 74
nucleus, 27
platelet production, 28
bone marrow, 72
demarcation membrane system, 72
mechanism and site of platelet release, 28–9
peripheral circulation, 73
ploidy, 71–2
pathophysiology and, 74, 75, 76
prostacyclin receptor expression, 149
virus interactions, 807–9
megakaryocytopenia
acquired amegakaryocytic thrombocytopenic purpura (AATP), 532
congenital amegakaryocytic thrombocytopenia (CAMT), 523, 537, 566
TAR-syndrome (thrombocytopenia with absent radius), 523–4, 537, 566
megakaryopoiesis, 31–5
clinical trials, 35
GPIb–IX–V role, 184
growth promoting cytokines, 34
microenvironment and, 34
negative regulators, 34
preclinical studies, 34–5
thrombopoietin biological activities, 33–4
thrombopoietin production and regulation, 32–3
see also megakaryocyte (MK); thrombopoietin (TPO)
megaloblastic anemia, 533–4
meizothrombin, 382
melanoma, 828
membrane complexes (MC), 42, 64–5
membrane ruffles, pleckstrin and, 276
membrane scrambling, 385
meningitis, 786
menstrual cycle, 478
cyclic thrombocytopenia and, 537
metal ion-dependent adhesion site (MIDAS), 81–2, 161, 190, 191, 308, 643–6
metastasis, 824–6, 827–9
immune response to, 829–30
inhibition of, 830–1
integrin αIIbβ3 role, 827–8
selectin role, 828–9
see also malignancy
8-methoxypsoralen (8-MOP), 810–11
microfilaments *see* actin filaments
microparticles, 11, 385, 492–3
microthrombocytopenia, X-linked recessive, 515–17
microtubules, 56–8, 94–5
platelet activation and, 96
platelet secretion and, 362
microvesicles, 47, 229
detection of, 465–6
formation of, 360
migraine, 900
mitochondria, 104
mitogen activated protein kinases (MAPKs), 226–7, 417
MNK1, 227, 228
moesin, 328
monoclonal antibodies
flow cytometry, 486–7, 489–90
to αIIbβ3 (GPIIb–IIIa), 831, 913
to TNFα, 912
monoclonal antibody immobilization of platelet glycoprotein assay (MAIPA), 557, 575
monocyte chemotactic proteins (MCPs), 395, 872–3
atherosclerosis and, 738, 739
monocytes, 869
platelet interactions during inflammation, 872–3
role in coagulation, 415–16
vascular disease and, 421
see also leukocytes
Montreal platelet syndrome, 348, 521
MPLV (myeloproliferative leukemia virus), 31
multimerin, 522, 662
myelodysplastic syndrome (MDS), 532, 533, 534–6, 696
cytogenetic abnormalities, 535
cytokine role, 535
differential diagnosis, 627
environmental carcinogens, 535
genetic defects, 535
genetic predisposition, 534–5
immune mechanisms, 535–6
treatment, 536
myelofibrosis, 566, 628, 629, 633
myeloproliferative disorders, 695–6
thrombocytosis and, 633, 695
see also specific disorders
myocardial infarction, 421, 734–5, 743
as outcome in clinical trials, 1007
aspirin therapy benefits, 920, 922, 923, 965, 1013
integrin α2 allele relationship, 768

Cambridge Books Online © Cambridge University Press, 2009

platelet characteristics and, 745
platelet heterogeneity, 74–5, 76
polymorphonuclear leukocytes and, 419, 420, 421
primary prevention, 923, 1013
secondary prevention, 1014
see also acute coronary syndromes
myosin, 14
myosin light chain kinase (MLCK), 274, 296, 297, 325, 396
myosin light chain phosphatase (MLCP), 325
myosin light chain phosphorylation, 274
platelet shape change and, 324–5, 328

N-ethylmaleimide-sensitive fusion protein (NSF), 362–4
Na^+/Ca^{2+} exchange, 267
Na^+/H^+ exchanger, 210
Naftidrofuryl (NAF), 330
naproxen, 955
natural killer (NK) cells, 829–30
nedocromil sodium, 995
Neisseria meningitidis, 786
neonatal alloimmune thrombocytopenia (NAIT), 551, 561–2, 569
platelet transfusion, 562, 577, 579, 713–14
see also alloimmune thrombocytopenia
nephrotic syndrome (NS), 841–2
neurons, 894
serotonin reuptake, 896–7
neurotransmitter release, 362
neutrophil-activating peptide (NAP-2), 415
neutrophil–platelet interactions, 869–70, 871–2, 874, 875, 876
nitric oxide (NO), 13–14, 293, 432, 738
atherosclerosis and, 439–40, 441, 443, 744
endothelial dysfunction and, 439–40, 443, 746
impaired NO bioavailability, 440
lipid peroxidation, 440
inhibition of platelet activation, 348, 435–7, 732
cGMP-independent mechanisms, 437
inhibition of platelet aggregation, 13–14, 443–4
platelet-derived, 443–4
uremia and, 839
vasodilatory activity, 433–4
nitric oxide synthase (NOS), 433–4, 436
gene disruption, 443
gene transfer, 984–5
migraine and, 900
non-steroidal anti-inflammatory drugs (NSAIDs), 690, 840, 955, 995–6
inflammatory bowel disease and, 911
NSAID-sensitive asthma, 861
see also aspirin
NSP-513, 959
nuclear factor kappa B (NFκB) transcription factors, 739
nucleus, 27

obsessive compulsive disorder, 901
omeprazole, 926
OPC-33540, 959
open canalicular system (OCS), 41–2, 43–4, 47, 50–4, 61–3, 64–5
oral contraceptives, 478
orbofiban, 949
organelles, 59–61, 104–10
mitochondria, 104
see also alpha-granules; dense bodies/granules; lysosomes
osteogenesis imperfecta, 682
osteonectin, 108
oviduct, platelet activating factor actions on, 889
oxidative stress, 440, 478, 745

P2X receptor, 129, 261–2, 664
agonists, 131
antagonists, 131
defects, 133, 358, 678
role in platelet function, 135, 342
see also ADP receptors
P2Y receptors, 127, 128–9, 130, 664, 973
agonists, 131
antagonists, 131, 930, 932, 964
as antithrombotic drug targets, 135
congenital defects, 133, 664–5, 678
identification of, 129, 973
role in platelet function, 133–5, 209–10, 342, 930
see also ADP receptors
p47 collagen receptor, 163
p65 collagen receptor, 163
p85 collagen receptor, 163
P-selectin, 11, 107, 108, 346, 358, 416–17
as laboratory marker
for activated platelets, 465, 486–7, 491, 755
for thrombosis, 755, 756, 757
atherosclerosis and, 740–1
blockade, 422
chemotaxis and, 393–4, 397, 405
inflammation and, 874–5, 992
inflammatory bowel disease and, 910
malignancy and, 828–9
platelet–leukocyte interaction and, 416, 740–1
adhesive mechanisms, 416–17
asthmatics, 859
vascular damage and, 419–20
vascular disease and, 421
P-selectin glycoprotein ligand 1 (PSGL1), 108, 358, 394, 397, 828, 875
platelet–leukocyte adhesive mechanisms, 417–18, 859
panic attack, 901
parasites, 815–21
platelet stimulation against, 816–19
C3b, 818
C-reactive protein, 817
diethylcarbamazine, 819
IgE, 816–17
interleukin-6, 818
lymphocyte mediators, 817–18

Cambridge Books Online © Cambridge University Press, 2009

parasites (*cont.*)
platelet stimulation against (*cont.*)
platelet activating factor (PAF), 818–19
platelet derived growth factor, 817
substance P, 817
thrombin, 818–19
thrombocytopenia and, 815–16
parathyroid hormone (PTH), 838
Paris-Trousseau thrombocytopenia, 522, 566, 662
paroxetine, 898
paroxysmal nocturnal hemoglobinuria (PNH), 532–3
parvovirus B-19, 808–9
Pasteurella haemolytica, 789
penicillin, 690
pentoxifylline, 1055–6
peripheral vascular disease (PVD), 330, 1053–9
antiplatelet therapy, 1054–9
arterial surgery and, 1056–7
future prospect, 1059
limb ischemic symptoms and, 1055–6
percutaneous endovascular revascularization and, 1057–9
prevention of vascular events, 1054
pernicious anemia, 534
peroxynitrite, 440
petechiae, 546, 573
PFA-100 in vitro platelet function analyser, 461
antiplatelet therapy monitoring, 474
pharmacogenetics, 966–7
ADP receptor, 973
proteomics, 974–5
thienopyridines, 972–3
Thrombosis Prevention Trial reinterpretation, 970–2
variability in drug response, 967–73
abciximab, 969–70
aspirin, 970
thromboxane synthase inhibitors, 968–9
warfarin, 967–8
see also gene therapy
phosphatidic acid (PA), 239, 249, 253
cyclic AMP effect, 297
phospholipase C activation, 246
phosphatidylcholine, 221–2, 229, 249
phosphatidylethanolamine, 221–2, 229, 382
phosphatidylinositol, 222
allergic diseases and, 852
defects in metabolism, 680
phosphatidylinositol 3,4,5-trisphosphate (PIP_3), 238
phosphatidylinositol 3-kinase (PI-3K), 238, 247, 285, 329
phosphatidylinositol 4,5-bisphosphate (PIP_2), 14, 99, 238, 329
phospholipase D regulation, 250
phosphatidylinositol 4-phosphate (PIP), 14
phosphatidylserine, 229, 231, 360, 382
phosphodiesterases (PDE), 294–5, 958–9
cyclic AMP regulation, 295–6, 958–9
inhibitors, 958–9
PDE2 family, 295
PDE3, 295
PDE5, 295
phosphoinositide-3-kinase (PI3-kinase), 436
phosphoinositides, 14, 114, 265
defects in metabolism, 680
platelet shape change and, 98, 99, 100, 328–9
phospholipase A_2 (PLA_2), 114, 221–32, 369, 372, 993
cytosolic $cPLA_2$, 224, 372
arachidonic acid release and, 372
regulation by calcium, 225
regulation by phosphorylation, 226–8
group IIA $sPLA_2$, 224, 228–32
anticoagulant effect, 230–1
antimicrobial effect, 231–2
arachidonic acid release and, 228–9, 372
degradation of apoptotic and injured cells, 231
role in atherogenesis, 231
intracellular calcium-independent $iPLA_2$, 224
pancreatic $sPLA_2$, 224
thromboxane A_2 synthesis and, 372
phospholipase C (PLC), 98, 99, 114, 238–49
activation in platelets, 239–40, 243–8, 273, 286
defects, 679–80
agonist-induced activation in platelets, 239–40
bacterial, 787–8
cytoskeleton and, 248–9
isozymes, 240–1
structure, 241–3
C2 domain, 243
C-terminal region, 243
catalytic domain, 241
EF-hand motifs, 243
pleckstrin homology (PH) domain, 241–3
X/Y linker region, 243
phospholipase D (PLD), 249–53
isozyme structures, 251–2
regulation, 250–1, 252–3
by low molecular weight G-proteins, 250–1
by PIP_2, 250
by protein kinase C, 251
in platelets, 252–3
phosphorylation, 281
cyclic AMP and, 296–7
cyclic GMP and, 298
G proteins, 274
myosin light chain, 274
platelet shape change and, 324–5, 328
phospholipase A_2 regulation, 226–8
phospholipase C regulation, 246–8
pleckstrin, 276–7
protein kinase C, 274
thromboxane receptor, 274
tyrosine phosphorylation events in human platelets, 286–7
inside–out signalling, 286

Cambridge Books Online © Cambridge University Press, 2009

outside–in signalling, 286–7
platelet shape change and, 325–6
see also specific kinases
piceatannol, 422
picotamide, 956–8
plasma membrane Ca^{2+}-ATPase (PMCA), 267–8
plasmin, 346, 691–2
platelet aggregation and, 347
plasmin–plasmin inhibitor complex (PPIC), 785
plasminogen activator (PA), 438
tissue plasminogen activator (t-PA), 503, 691, 739, 991
gene restoration of, 981
plasminogen activator inhibitor 1 (PAI 1), 107, 359, 739, 948
endothelial dysfunction and, 438
inhibition of vascular smooth muscle cell migration, 984
Plasmodium berghei, 815–16
Plasmodium falciparum, 816
Plasmodium vivax, 815
platelet activating factor (PAF), 8, 9, 400, 405, 993
actions on oviduct, 889
allergic diseases and, 852–4, 855, 858
embryo-derived, 885–6
autocrine actions of, 890
physiological actions of, 888
glomerular diseases and, 842
inflammatory bowel disease and, 910
platelet aggregation and, 345–6
platelet stimulation against parasites, 818–19
reproductive tract, 886–8
role in early pregnancy, 888–90
platelet activating factor–acetyl hydrolase (PAF–AH), 746
platelet activation, 45–54, 95, 113–14, 127–8, 166, 459–60
ADP and, 929–30
allergic diseases and, 852–4
atherothrombosis and, 1039–40
cytoskeleton and, 95–8
dense granule role, 359–60
detection of circulating activated platelets, 465
G protein role, 210–11
inflammation and, 871
inflammatory bowel disease and, 910–11
inhibition of, 732
cyclic AMP, 296, 732
cyclic GMP, 298
nitric oxide, 435–7, 732
polymorphonuclear leukocytes (PMNs), 414
prostaglandins, 290, 348, 375, 435
leukocyte-induced, 412–14
markers of, 324, 486–7, 745
measurements, 464–5, 745
platelet hyper-reactivity, 491
platelet hyporeactivity, 492
role in early pregnancy, 888–9
thrombin and, 47–8, 51–3, 99, 113–14, 994
thrombosis and, 728–30, 744–5
tyrosine phosphorylation events, 286–7
see also platelet aggregation; platelet receptors; platelet shape change
platelet activity suppressive lymphokine (PASL), 818
platelet adhesion, 183, 304–14
adhesive proteins, 307–10
collagens, 308–9
fibrinogen/fibrin, 309–10
fibronectin, 309
laminin, 310
von Willebrand factor, 307–8, 310, 459, 744, 838
antiplatelet therapy monitoring, 477
defects, 674–6
see also Bernard–Soulier syndrome; von Willebrand's disease
general mechanism, 304–6
genetics of, 312
inflammatory cells *see* inflammation
platelet–leukocyte adhesive mechanisms, 416–19, 859
see also leukocytes
receptors and, 310–12
fibronectin receptor, 311–12
GPIb–V–IX complex, 310–11
GPIIb–IIIa, 311
GPVI, 311
integrin $\alpha2\beta1$, 311
shear stress influence, 306–7
thrombocytopenia and, 312
thrombosis and, 729
uremia and, 838–9
see also collagen; thrombosis; thrombus formation
platelet aggregation, 6–10, 127, 183, 338–49, 459–60, 1063
aggregating agents, 338
ADP, 133–5, 342–3, 444, 462, 929–30, 1064–5
arachidonic acid, 344
arginine vasopressin, 346
bacterial *see* bacterial infections
calcium, 896
cathepsin G, 346–7, 413–14
collagen, 343–4, 462
epinephrine, 345, 678–9
platelet activating factor (PAF), 345–6
serotonin (5-HT), 346, 444, 895, 1062
thrombin, 344–5, 439, 472
thrombospondin, 346
thromboxane A_2, 344, 413, 444
allergic diseases and, 852
dense granule role, 105–6
detection of circulating platelet aggregates, 466
disorders, 349, 676, 678–9
fibrinogen role, 188, 192, 941–2
GPIIb–IIIa complex role, 459–60, 940–3
antagonist effects, 944–5
inside-out signalling, 940–1
outside-in signalling, 941–2
platelet agonist receptors, 942–3

Cambridge Books Online © Cambridge University Press, 2009

platelet aggregation (*cont.*)
  in vitro, 339–42, 349
    anticoagulant effects, 340–2
    impedance platelet aggregometry, 340
    turbidometric platelet aggregation, 339–0
  inflammatory bowel disease and, 908–9, 910–11
  inhibition of, 10, 339, 929, 1065
    adenosine, 348
    aspirin, 8, 12, 65
    cyclic GMP, 13–14
    endogenous inhibitors, 348–9
    nitric oxide, 13–14, 443–4
    prostaglandins, 12–13, 348
    ticlopidine/clopidogrel, 929
  leukocyte interaction and, 413
  plasmin effects, 347
  platelet hypersensitivity, 348, 841–2
  protein kinase C role, 274, 275
  relevance to neurotransmission, 894–6
  shear-induced, 348
  smoking effects, 478
  spontaneous aggregation, 348
  storage pool disease and, 658–9
  tests of *see* platelet function tests
  thrombosis and, 729–30
  tumour cell-induced, 826
  tyrosine phosphorylation events, 286–7
    inside–out signalling, 286
    outside–in signalling, 286–7
  uremia and, 838, 841–2
  see also agglutination; platelet activation; thrombus formation
platelet aggregation-associated protein (PAAP), 784, 785, 787
platelet basic protein (PBP), 827
platelet concentrates (PC), 707–9
  apheresis PC (AP-PC), 707
  buffy coat method PC (BC-PC), 708
  platelet-rich-plasma PC (PRP-PC), 707
  storage, 709–10
    contaminating microorganisms, 718, 809–10
    experimental approaches, 710
    frozen storage, 710
    liquid storage, 709–10
    pathogen inactivation, 809–12
platelet count, 474, 542
  atherothrombosis and, 744–5
  essential thrombocythemia diagnosis, 626
  fetal, 579
  inflammatory bowel disease and, 908, 910
  see also thrombocytopenia
platelet density
  heterogeneity, 70
  myocardial infarction and, 74
  platelet ageing and, 71
platelet derived collagenase inhibitor (PDCI), 107
platelet derived growth factor (PDGF), 108, 403, 415, 566, 738, 826, 993
  inflammatory bowel disease and, 911
  platelet stimulation against parasites, 817
  role in asthma, 858
  role in glomerular diseases, 843
platelet derived histamine releasing factor (PDHRF), 856
platelet derived hyper-responsiveness factor (PDHF), 858
platelet endothelial cell adhesion molecule-1 (PECAM-1), 165, 405, 739
  bacterial interaction, 789
platelet factor 3 (PF3), 55
  see also platelet procoagulant activity (PCA)
platelet factor 4 (PF4), 107, 108, 398, 404, 993
  as laboratory marker for thrombosis, 755
  asthma and, 856
  heparin-induced thrombocytopenia and, 591–2
  inflammatory bowel disease and, 909–10
  leukocyte stimulation, 415
  malignancy and, 827
  measurement, 464, 755
  megakaryopoiesis inhibition, 34
platelet function tests, 460–7, 471
  antiplatelet therapy monitoring, 471–9
  global platelet functions, 460–2
    bleeding time, 460–1, 477
    clot signature analyser, 461–2, 478
    PFA-100 in vitro platelet function analyser, 461, 474
  platelet activation marker measurement, 464–5, 486–7
    β-thromboglobulin and platelet factor-4, 464
    glycocalicin, 465
    soluble P-selectin, 465, 486–7
    urinary thromboxane metabolites, 464–5, 476
  platelet aggregation tests, 462–4, 471, 473–4
    agonist-induced aggregation, 462–3
    anticoagulant, 474
    platelet functions under flow, 464
    rapid platelet function assay (RPFA), 463, 474–5, 945
    reliability, 474
    ristocetin-induced agglutination, 463
    selectivity, 474
    shear-induced aggregation, 463–4
    spontaneous aggregation, 462
    thrombocytopenia, 473–4
    whole blood aggregation, 463, 475
  platelet gene polymorphism test, 466–7
  platelet procoagulant activity, 466
  see also flow cytometry
platelet microbicidal proteins (PMPs), 789–90, 791
platelet procoagulant activity (PCA), 11, 381, 383–7
  fibrin role, 387–9
  measurement, 466
  thrombin role, 387–9
  see also coagulation; platelet factor 3 (PF3)
platelet production, 72–4
  bone marrow disorders and *see* bone marrow
  cellular aspects, 25–30
  heterogeneity and, 71–2, 74

Cambridge Books Online © Cambridge University Press, 2009

regulation, 31–5, 71, 75, 528–9
disorders, 536–7
site of, 72–4
bone marrow, 72
demarcation membrane system (DMS), 72
lungs, 73–4
peripheral circulation, 73
see also megakaryocyte (MK)
platelet receptors, 8, 28, 47–54
adenosine receptors, 292
adenylyl cyclase activation and, 290–2
adenylyl cyclase inhibition and, 292–3
adhesion receptors, 80–8
ADP receptors, 128–35, 292
bacterial interactions, 788–9
collagen receptors, 160–9
epinephrine receptor, 293
fibrinogen, 188–95
mobility, 47–54
multiple agonist receptors, 942–3
prostacyclin receptor (IP), 148–50, 290
prostaglandin D_2 receptor (DP), 152, 292
prostaglandin E_2 receptors (EPs), 150–2, 290–2
protease-activated receptors, 113–21
thrombin receptors, 113, 121–2, 293, 413–14
thromboxane A_2 receptor (TP), 140–8, 293
von Willebrand factor, 179–85
see also specific receptors
platelet sec I protein (PSP), 361, 364
platelet secretion, 357–65, 655–6
defects, 656–66, 676
alpha granule defects, 661–3
classification, 656–7
definition, 656, 665
dense granule defects, 657–61, 662–3
primary secretion defects, 665–6, 677–81
therapy, 666
see also alpha-granules; dense bodies/granules; lysosomes
platelet shape change, 319–31, 1064
definitions, 320
disease and, 329–30
morphological shape changes, 321–4
new surface structures, 323–4
volume increases, 323
optical shape change, 320–1
study using calcium chelating agents, 321
relevance to neurotransmission, 895
signalling and, 14, 98–100, 324–9
actin-modulating proteins and, 326–8
calcium role, 98, 99–100, 324
G protein role, 325
kinetics, 328
myosin light chain phosphorylation, 324–5
phosphoinositides and, 98, 99, 100, 328–9
platelet inhibitor effects, 328
protein kinase C role, 326
small GTPase role, 100, 325
tyrosine phosphorylation and, 325–6
speed of, 321–2
platelet storage *see* platelet concentrates
platelet suspension immunofluorescent test (PSIFT), 575
platelet transfusion, 6, 683
cardiopulmonary bypass and, 713
clinical response to, 711–13
platelet dose and, 711–12
platelet transfusion trigger, 712–13
thrombocytopenia secondary to bone marrow failure, 711
complications, 715–18
alloimmunization to class I HLA antigens, 715–17
contaminating microorganisms, 718, 809–10
contaminating red cells, 718
cytomegalovirus transmission, 718
febrile and allergic reactions, 717–18
graft-vs.-host disease, 718
refractory patients, 716–17
contraindications, 715
hereditary thrombocytopenia, 714
idiopathic thrombocytopenic purpura, 713
massive red cell transfusion and, 713
neonatal alloimmune thrombocytopenia, 562, 577, 579, 713–14
platelet preparation, 707–9
see also platelet concentrates
post-transfusion purpura (PTP), 551–2, 561, 714
qualitative platelet disorders, 714–15
refractoriness to, 552
safety, 718, 807, 809–10
virus inactivation, 810–12
splenomegaly and, 713
platelets, 1061–2
activation *see* platelet activation
adhesion *see* platelet adhesion
aggregation *see* platelet aggregation
chemotaxis
animal studies, 400–2
chemotactic effects on other cells, 403–5, 415
human studies, 402
in vitro, 398–400
in vivo, 400–2
migratory ability, 397–8
collagen interactions *see* collagen
dendritic platelets, 45–6, 47
destruction of, 75
endothelium and, 4, 184, 739–40, 744
see also platelet adhesion
heterogeneity, 70–6
pathophysiology, 74–5
platelet ageing as cause, 71
thrombopoiesis as origin, 71–2
history of research, 7
human platelet antigens (HPA), 570–1
alloimmunization, 571–3
imaging, 757

Cambridge Books Online © Cambridge University Press, 2009

platelets (*cont.*)
internal membrane system, 61–5
dense tubular system (DTS), 41–2, 63–5
membrane complexes (MC), 42, 64–5
surface-connected open canalicular system (OCS), 41–4, 47, 50–4, 61–5
isolation of, 6
leukocyte interactions *see* leukocytes
lifespan of, 6
organelle zone, 43, 59–61
see also organelles
peripheral zone, 41–2, 43–56
exterior coat, 41, 43–54
submembrane region, 55–6
unit membrane system, 41–2, 54–5
production of *see* platelet production
release reaction, 10–11
reticulated, 492
sol–gel zone, 42–3, 56–9
microfilaments, 58–9
microtubules, 56–8
source of, 5–6
spreading, 45–6, 95–6, 277
transfusion of *see* platelet transfusion
tumour cell interactions *see* malignancy
vessel injury and, 4–5
viral interactions *see* viral infection
pleckstrin, 276
actin organization and, 276–7
integrin-dependent cell spreading and, 277
membrane ruffle formation, 276
phosphorylation defects, 679–80
protein kinase C and, 276–8
polycythemia vera (PV), 629
differential diagnosis, 627
thrombocytosis in, 633
polymorphism, 966
CYP2CP gene, 967–8, 972
glycoproteins, 312, 466–7, 479
GPIb, 643, 765–6
GPIb–IX–V complex, 181
GPIIb–IIIa complex, 771–3, 969–70
integrins, 312, 766, 767–8
single nucleotide polymorphism (SNP), 466–7, 966, 967
test of, 466–7
variable numbers tandem repeat (VNTR) polymorphism, 467
warfarin safety and, 967–8
polymorphonuclear leukocytes (PMNs), 394, 404, 993
arachidonic acid metabolism, 416
inhibition of platelet activation, 414
myocardial infarction and, 419, 420, 421
platelet aggregation induction, 413
platelet–PMN adhesive mechanisms, 417–19, 859
stimulation of by platelets, 415
vascular damage and, 420
see also leukocytes
polyphosphoinositides *see* phosphoinositides
Porphyromonas gingivalis, 785, 791
post-transfusion purpura (PTP), 551–2, 561, 714
PPACK, 340
PRAK1, 227
pregnancy, 885–91
aplastic anemia and, 532
essential thrombocythemia and, 632–3
heparin-induced thrombocytopenia therapy, 599
idiopathic thrombocytopenic purpura and, 549–50, 558
platelet activation role, 888–9
preimplantation phase, 885
thrombocytopenia and, 885–6
see also embryo-derived platelet-activating factor
primary secretion defects, 665–6, 677–81
calcium mobilization defects, 679–80
GPIIb–IIIa activation defects, 680–1
GTP-binding protein activation defects, 679
phospholipase C activation defects, 679–80
pleckstrin phosphorylation defects, 679–80
receptor defects, 677
ADP receptors, 664–5, 678
collagen receptor, 677–8
epinephrine response impairment, 678–9
thromboxane A_2 receptor, 663–4, 677
procoagulant activity *see* platelet procoagulant activity (PCA)
profilin, 93, 238, 249
prostacyclin (PGI_2), 13, 148, 149, 223, 435, 738, 981
allergic diseases and, 856
aspirin pharmacodynamics, 920
endothelial dysfunction and, 439
inhibition of platelet activation, 348, 375, 435, 476, 738
intravenous infusion, 691
synthesis, 140, 435, 873, 981
uremia and, 839
vasodilatory activity, 432–3, 439
prostacyclin receptor (IP), 148–50
adenylyl cyclase stimulation, 290
regulation, 149
signal transduction, 149
structure, 148–9
prostaglandin D_2 receptor (DP), 152, 292
regulation, 152
signal transduction, 152
structure, 152
prostaglandin E_2 receptors (EPs), 150–2
EP2, 290–2
EP3, 292
EP4, 292
regulation, 151–2
signal transduction, 151
structure, 150–1
prostaglandin H synthases, 369, 956
prostaglandins (PG), 12–13, 140
inhibition of platelet activation, 290
peripheral vascular disease therapy, 1056

Cambridge Books Online © Cambridge University Press, 2009

PGD$_2$, 140, 152, 223
PGE$_1$, 12–13, 140
allergic diseases and, 856
intravenous infusion, 691
PGE$_2$, 13, 140, 151–2, 223, 405
platelet aggregation and, 347
PGF$_2$, 13, 223
PGG$_2$, 13, 140, 222–3
PGH$_2$, 13, 140, 222–3, 432, 956
vasoconstriction, 433, 435
receptors *see specific receptors*
synthesis, 65, 140, 370, 373–4
prostanoids, 140, 369
biosynthesis, 369–71
see also prostacyclin; prostaglandins; thromboxane A$_2$
protease nexin-2/amyloid beta-protein precursor (PN-2/APP), 107, 108
protease-activated receptors (PAR), 8, 99, 113–21, 414, 958, 992, 994
adenylyl cyclase inhibition, 293
genetics, 116
inactivation, 117–18, 958
interactions with proteases other than thrombin, 121
ligand binding domain, 117
mice, 118–19
platelets and, 118–21, 183, 344
receptor cleavage and recycling, 120–1, 183
receptor location, 119–20
replacement of, 117–18, 121
tissue distribution, 116–17
protein A, 787
protein C, 349, 382, 438, 870, 991–2, 1040
activated protein C (APC), 991, 992, 1040
protein disulphide isomerase (DPI), 164
protein kinase A (PKA), 146–7, 293–4, 296
prostacyclin receptor interaction, 149
protein kinase AKT, 436
protein kinase C (PKC), 99, 146–8, 238, 272–8
actin organization and, 275–7
activation of, 273–4, 360
adenylyl cyclase and, 293
functions, 274–5
platelet aggregation, 274, 275
platelet secretion, 274, 275, 360–1
isozymes, 272–3
megakaryocyte differentiation and, 277–8
phospholipase D activation, 251
platelet shape change and, 326
protein kinase G (PKG), 296
deficient mice, 298
protein phosphorylation *see* phosphorylation
protein S, 438
deficiency, 439
protein tyrosine kinases *see* tyrosine kinases
proteinuria, 842–3
proteoglycans, 107
proteomics, 974–5
prothrombin, 382, 383
receptor, 386
prothrombinase, 382, 383, 1040
pseudopod formation, 45–7, 321
pseudothrombocytopenia, 542, 589–91
PU.1 transcription factor, 761
pulmonary embolism (PE), 753, 757
aspirin therapy, 753–4
see also thrombosis
Pulmonary Embolism Prevention (PEP) Trial, 754
pulmonary vessels, platelet production, 73–4
purpura
acquired amegakaryocytic thrombocytopenic (AATP), 532
childhood
acute autoimmune thrombocytopenic purpura, 557, 558–61
chronic autoimmune thrombocytopenic purpura, 558, 560–1
therapy, 559–61
idiopathic thrombocytopenic (ITP), 32–3, 529, 544, 545–51, 694
immune thrombocytopenic (ITP), 32–3
post-transfusion (PTP), 551–2, 561, 714
see also thrombotic thrombocytopenic purpura
Pyk2 tyrosine kinase, 285

Quebec platelet disorder, 109, 522, 662, 676–7
quinidine-induced thrombocytopenia, 553
see also drug-induced thrombocytopenia

Rab GDP dissociation inhibitor (GDI), 364
Rab GTPase subfamily, 206, 207, 215
exocytosis and, 364–5
Rac GTP-binding protein, 329
radiation exposure, 531
randomization, 1004
randomized controlled trials *see* clinical trial design
ranitidine, 926
RANTES, 395, 398, 404–5, 827, 872
asthma and, 856
rapid platelet function assay (RPFA), 463
antiplatelet therapy monitoring, 474–5, 945
Ras GTPase subfamily, 206–7, 212–14
Raynaud's phenomenon, 869
reactive thrombocytosis (RT), 623, 624
differential diagnosis, 627
receptor induced binding sites (RIBS), 193, 486
receptor occupancy assay, 475–6
red blood cells
platelet interactions during inflammation, 873
transfusion
thrombocytopenia and, 713
uremia, 693
related adhesion focal tyrosine kinase (RAFTK), 285
renal disease
hemorrhagic fever with renal syndrome (HFRS), 808
renal failure, 837
thrombopoietin deficiency, 537
see also glomerular diseases; uremia

Cambridge Books Online © Cambridge University Press, 2009

renal vein thrombosis (RVT), 841–2
restenosis, 744, 1056
  vascular smooth muscle cell role, 982
reticulated platelets, 492
reticuloendothelial (RE) blockade, 547
RGD-containing peptides, 82, 194, 831, 964–5
Rho GTPase subfamily, 100, 206, 207, 211, 214
  cell migration and, 395
  phospholipase D regulation, 251
  platelet shape change and, 325, 329
rhodocytin, 165
Rickettsial infections, 783–4
ristocetin, 9–10
  agglutination induction, 348, 463
  antiplatelet therapy monitoring, 472
Rocky Mountain spotted fever, 784

sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), 267
schistosomes, 815, 816, 817
  immune mechanisms against, 819–20, 854, 855
  S. mansoni, 816, 817, 818, 854, 855
schizophrenia, 895, 900–1
Scott syndrome, 680
screening, alloimmune thrombocytopenia, 575–6
Sebastian platelet syndrome, 519, 565
secretion *see* platelet secretion
selectins, 393–4
  E-selectin, 393, 394
  L-selectin, 393, 394
  malignancy and, 828–9
  see also P-selectin
selective serotonin reuptake inhibitor (SSRI), 895, 901
sepsis, 992, 993
  see also bacterial infections
serotonin (5-HT), 9, 105–6, 403, 852, 856, 894, 1062
  5-HT receptors, 896, 956
  depression and, 895, 896–7
  inflammation and, 995
  platelet aggregation and, 346, 444, 895, 1062
  release of, 358
  reuptake changes, 896–7
    alcoholism, 902
    anxiety disorders, 901
    autism, 900
    headache, 900
    mania, 901
    schizophrenia, 901
  transporter properties, 896–7, 902
    3H-imipramine and 3H-paroxetine binding sites, 898, 902
  uremia and, 838
serotonin antagonists, 956
shape change *see* platelet shape change
shear stress, 306
  atherosclerosis and, 738
  influence on platelet adhesion, 306–7
  platelet aggregation induction, 348
    measurement, 463–4
signal transduction, 14, 98–100, 207, 471, 674, 731–2
  collagen receptors, 165–8, 283–4, 298
  congenital defects, 674, 677–83
    calcium mobilization defects, 679–80
    GPIIb–IIIa activation defects, 680–1
    GTP-binding protein activation defects, 679
    phospholipase C activation defects, 679–80
    pleckstrin phosphorylation defects, 679–80
    receptor defects, 663–5, 677–9
  cyclic AMP effects, 297
  GPIIb–IIIa in platelet aggregation, 940–2
    inside-out signalling, 940–1
    outside-in signalling, 941–2
  GPVI, 166–8
  inhibitors of, 473
  integrins, 168, 284
  prostacyclin receptor, 149
  prostaglandin receptors, 151, 152
  shape change and *see* platelet shape change
  thromboxane A_2 receptor, 145–6, 376
  through the GPIb complex, 765
  tyrosine phosphorylation events in human platelets, 286–7
    inside–out signalling, 286
    outside–in signalling, 286–7
single nucleotide polymorphism (SNP), 466–7, 966, 967
SLP-76 adaptor protein, 247
small GTPases, 100, 206–7
  Arf GTPase subfamily, 206, 207
    phospholipase D regulation, 250–1
  Rab GTPase subfamily, 206, 207, 215
    exocytosis and, 364–5
  Ras GTPase subfamily, 206–7, 212–14
  Rho GTPase subfamily, 100, 206, 207, 211, 214
    cell migration and, 395
    phospholipase D regulation, 251
    platelet shape change and, 325, 329
small heat-shock proteins (sHsps), 327
smoking, 478, 745
SNAP family, 363–4
SNARE hypothesis, 362–4
  SNARE family, 362–3
  SNAREs in platelets, 363–4
  v- and t-SNARE regulation, 363
sodium citrate, 341, 474
Sonoclot analyser, 478
spectrin, 94
sphingosine-1-phosphate (S1P), 212, 214, 829
splenectomy
  idiopathic thrombocytopenic purpura treatment, 548
    childhood, 560
  thrombocytosis and, 624–5
splenomegaly, 536, 543–4
  platelet transfusion and, 713

Cambridge Books Online © Cambridge University Press, 2009

spontaneous platelet aggregation (SPA), 462
spotted fevers, 784
spread platelets, 45–6, 95–6, 277
Src-family tyrosine kinases, 99, 281–3
collagen receptor interactions, 165, 166, 167, 169
platelet–leukocyte adhesive mechanisms, 417–19
stable angina, 734, 922
Staphylococcus aureus, 788, 790–1
bacterial ligands, 787–8
disseminated intravascular coagulation and, 786
infective endocarditis, 784
staphylokinase, 791
statins, 746
stem cell depletion, 530–3
see also bone marrow
stenosis
coronary arteries, 742–3
restenosis, 744, 1056
vascular smooth muscle cell role, 982
storage of platelets *see* platelet concentrates
storage pool disease (SPD), 11, 60, 106, 313, 657–9, 676–7
alpha/delta-storage pool deficiency, 662–3
clinical features, 657
diagnosis, 492, 659
frequency, 683
laboratory findings, 657–9
see also grey platelet syndrome
streptococci, 787, 788–9
infective endocarditis, 784–5
S. pneumoniae, 786
S. pyogenes, 785
S. sanguis, 784–90
streptokinase, 691, 1022
stress fibres, 395, 397
stress-activated protein kinase (SAPK) family, 226–7
stroke
antiplatelet therapy, 923, 1041
aspirin, 922–3, 1013, 1041, 1042–4, 1046–9
dipyridamole, 1046–8, 1049
GPIIb–IIIa antagonists, 1048
primary prevention, 1013
secondary prevention, 1041–9
thienopyridines, 1044–6, 1049
as outcome in clinical trials, 1007
definition, 1038
etiology, 1038
incidence/prevalence, 1038
pathogenesis of ischemic stroke, 1038–40
atherosclerosis, 1039
coagulation, 1040
normal endothelium, 1038–9
platelet recruitment into the thrombus, 1040
sequelae of plaque rupture, 1039–40
triggers of plaque rupture, 1039
unstable plaque, 1039
prognosis, 1038
stromal cell-derived factor-1 (SDF-1), 29, 396, 398, 739, 855
submembrane filaments, 55–6
substance P (SP), 817
sulfasalazine, 911
sulfinpyrazone, 955, 964
sulotroban, 958
superoxide anion, 440, 443
surface-connected open canalicular system (OCS), 41–4, 47, 50–4, 61–5
Syk tyrosine kinase, 87, 99, 246–7, 283–4, 286, 765
synaptobrevin, 363
syntaxin family, 361, 363–4
systemic lupus erythematosus, 532, 550–1, 694

TAR-syndrome (thrombocytopenia with absent radius), 523–4, 537, 566, 680
Tec family tyrosine kinases, 285
testosterone, 148
theophylline, 854
thienopyridines, 131–3, 343, 690–1, 929
pharmacogenetic approach, 972–3
pharmacology, 930–4
antithrombotic activity, 932–3
clinical pharmacology, 933–4
metabolism and pharmacokinetics, 930–2, 972
mode of action on platelets, 932, 972
side effects, 972–3
stroke secondary prevention, 1044–6
see also clopidogrel; ticlopidine
thioredoxin, 889
thrombasthenia, 680, 683, 715
see also Glanzmann thrombasthenia
thrombin, 8, 113, 1040
antiplatelet therapy monitoring, 472
antithrombins, 349, 438, 958
binding to GPIb, 183–4
endothelial dysfunction and, 438–9
generation of, 381, 382–3
platelet role in, 383–7
heparin-induced thrombocytopenia and, 593–4
importance in thrombosis, 439, 733, 734
thrombin-induced clot formation model, 504–5
inflammation and, 870, 992
megakaryopoiesis inhibition, 34
phospholipase C activation, 244
platelet activation, 47–8, 51–3, 99, 113–14, 994
tyrosine phosphorylation events, 285, 286, 287
platelet aggregation and, 344–5, 439, 472
platelet procoagulant activity stimulation, 387–9
protein kinase C activation, 274
tyrosine kinase activation, 282–3, 284, 286
thrombin receptor activating peptides (TRAP), 99, 344–5
adenylyl cyclase inhibition, 293
antiplatelet therapy monitoring, 472, 479, 945

Cambridge Books Online © Cambridge University Press, 2009

thrombin receptors, 113, 121–2, 413–14, 958, 994
adenylyl cyclase inhibition, 293
antagonists, 958
see also protease-activated receptors (PAR)
thrombin–antithrombin complex (TAT), 785
thrombocythemia, 623
see also essential thrombocythemia (ET)
thrombocytopenia, 696
acquired amegakaryocytic thrombocytopenic purpura (AATP), 532
alcohol abuse and, 531
autosomal dominant thrombocytopenia, 518–21
macrothrombocytopenia, 518–21
thrombocytopenia with normal platelet size, 521–2
autosomal recessive thrombocytopenia, 522–4
macrothrombocytopenia, 522
thrombocytopenia with normal platelet size, 523–4
bone marrow disorders and *see* bone marrow
cardiopulmonary bypass and, 694, 713
childhood *see* childhood thrombocytopenia
congenital amegakaryocytic thrombocytopenia (CAMT), 523, 537, 566
cyclic thrombocytopenia, 537
disseminated intravascular coagulation and, 786
drug-induced *see* drug-induced thrombocytopenia; heparin-induced thrombocytopenia
immune-mediated thrombocytopenia, 542–53
alloimmune thrombocytopenia, 551–2, 561–2, 569–80, 694
autoimmune thrombocytopenia, 537, 545–51
general approach, 542–4
idiopathic thrombocytopenic purpura (ITP), 32–3, 529, 544, 545–50, 694
secondary immune thrombocytopenias, 550–1
treatment, 544, 547–9
see also drug-induced thrombocytopenia
massive red cell transfusion and, 713
Mediterranean thrombocytopenia, 566
parasite infection and, 815–16
Paris-Trousseau thrombocytopenia, 522, 566
platelet adhesion and, 312
platelet aggregometry and, 473–4
pregnancy-induced, 885–6
TAR-syndrome (thrombocytopenia with absent radius), 523–4, 537, 566, 680
tumour cell-induced, 825
uremia and, 838
viral infection and, 531, 550
X-linked recessive thrombocytopenia, 515–18
macrothrombocytopenia, 517–18
microthrombocytopenia, 515–17
thrombocytosis
classification, 623–4
definition, 623
inflammatory disease and, 624, 869
inflammatory bowel disease, 908
iron-deficiency anemia and, 625
malignancy and, 624
myeloproliferative disorders and, 633, 695
reactive thrombocytosis (RT), 623, 624
differential diagnosis, 627
splenectomy and, 624–5
thromboelastography, 478
thrombomodulin (TM), 991, 992, 1040
gene transfer, 980
thrombopoiesis *see* platelet production
thrombopoietin (TPO), 6, 32, 72, 311, 523, 528
biological activities, 33–4
clinical trials, 35
decreased sensitivity to, 537
deficiency, 536–7
autoimmune thrombocytopenia, 537
liver disease, 536–7
renal disease, 537
discovery of, 31
gene, 32
platelet volume and, 75
preclinical studies, 34–5
production and regulation, 32–3, 528–9, 623–4
protein kinase C and, 277–8
thrombocytopenia and, 523–4, 537, 556–7, 623–4
thrombocytosis/thrombocythemia and, 623–4
thrombosis, 3–4, 80, 460, 727–35, 978, 1063, 1064–5
acute thrombosis, 728
antithrombotic activity
aspirin, 922
ticlopidine and clopidogrel, 932–3
antithrombotic gene therapy, 978–82
cyclooxygenase-1 gene transfer to sites of arterial injury, 981–2
gene restoration of tissue factor pathway inhibitor, 982
gene restoration of tissue plasminogen activator, 981
gene transfer techniques, 980
thrombomodulin gene transfer, 980
arterial thrombosis, 4, 5, 727–35
essential thrombocythemia and, 628–9
hypercoagulable-dependent thrombosis, 734
lesion classification, 727
models, 442–3, 499–509
arterial eversion graft model, 507–8
copper coil model of arterial thrombosis, 503–4
electrically induced coronary thrombosis, 505–7
Folts coronary thrombosis model, 442, 499–502
thrombin-induced clot formation, 504–5
myeloproliferative disorders and, 695
plaque disruption, 727–8, 742, 1066
active, 727–8
passive, 727
platelet heterogeneity and, 74
platelet involvement, 3–4, 728–30, 744–5, 753–7
clinical trials, 753–4

Cambridge Books Online © Cambridge University Press, 2009

laboratory markers, 755–7
platelet imaging, 757
platelet–collagen interaction importance, 160
platelet–leukocyte interaction and, 419–21
animal models, 419–20
epidemiological and clinical studies, 420–1
progression, 727
risk factors, 745–6
thrombin importance, 439, 733, 734
tissue factor and, 732–3
uremia complications, 840–2
vasoconstriction and, 734
vein grafts and, 985
von Willebrand factor importance, 438, 460
see also atherothrombosis; deep vein thrombosis (DVT); pulmonary embolism (PE); thrombus formation
thrombospondin (TSP), 108, 415
malignancy and, 827
platelet aggregation induction, 346
TSP1, 195, 358
thrombosthenin, 14
thrombotic microangiopathies (TMA), 619
see also thrombotic thrombocytopenic purpura (TTP)
thrombotic thrombocytopenic purpura (TTP), 329, 610
antiendothelial cell antibodies and, 617–18
antiplatelet antibodies and, 617–18
apoptosis and, 618–19
childhood, 563
epidemiology, 610
pathogenesis, 611–13, 616–17
platelet aggregating agents in, 611
platelet transfusion and, 715
von Willebrand factor and, 611
vWF-cleaving protease deficiency, 612–13, 616–17
thromboxane A_2 (TxA2), 9, 140, 405, 993, 1065–6
allergic diseases and, 856
cyclic flow variants and, 501–2
G protein interactions, 211
measurement of urinary metabolites, 464–5
phospholipase C activation, 244, 248
platelet activation, 113–14, 221
platelet aggregation and, 344, 413, 444
synthesis, 140, 222–3, 370–5, 435
alteration of in humans, 374–5
aspirin pharmacodynamics, 920
aspirin-insensitive biosynthesis, 376–7
cyclooxygenase role, 372, 374, 375, 376–7, 435
endogenous modulation, 375
impairment, 373, 663, 681–2, 683, 838
phospholipase role, 372
uremia and, 838
see also thromboxane A_2 synthase (TXAS)
vasoconstriction induction, 435
thromboxane A_2 receptor (TP), 140–8, 375–6
adenylyl cyclase inhibition, 293
antagonists, 473, 506–7, 956–8, 964
defects, 663–4, 677, 680
isoforms, 375–6
phosphorylation, 274
regulation, 146–8
signal transduction, 145–6, 376
structure, 140–4
thromboxane A_2 synthase (TXAS), 222–3, 373–4
deficiency, 373, 663, 681–2
endogenous modulation, 375
inhibitors, 373–4, 506, 956–8, 964
genetic variability in response, 968–9
inflammatory bowel disease therapy, 911
thromboxane B_2 (TXB2), 371–2, 374, 873
antiplatelet therapy monitoring, 476
thrombus formation, 80, 183, 188, 459
integrin αIIbβ3 role, 770–3
mural thrombi, 743–4
incorporation into atherosclerotic plaques, 744
prevalence, 743
vs. occluding thrombi, 743–4
platelet recruitment, 1040
platelet role, 728–30
thrombin-induced clot formation model, 504–5
see also coagulation; platelet aggregation; thrombosis
thymosin-β4, 93
tianeptine, 852
ticlopidine, 131, 135, 343, 690–1, 929, 964
antiplatelet therapy monitoring, 479
cost, 1046
glomerular disease treatment, 844
inflammatory bowel disease therapy, 912–13
non-ST elevation acute coronary syndrome therapy, 1017
percutaneous coronary intervention and, 1024–5
peripheral vascular disease therapy, 1054, 1055, 1058–9
pharmacogenetic approach, 972–3
pharmacology, 930–4
clinical pharmacology, 933–4
metabolism and pharmacokinetics, 930, 972
mode of action on platelets, 932, 972
secondary prevention, 1014
stroke, 1044–6
side effects, 933–4, 972–3, 1046
tirofiban, 589, 691, 943–4
non-ST elevation acute coronary syndrome therapy, 1017–19
percutaneous coronary intervention and, 1032
see also GPIIb–IIIa antagonists
tissue factor (TF), 382, 383, 386–7, 415–16, 439, 982, 991
activated platelets and, 741
endothelial dysfunction and, 439
thrombogenesis and, 732–3, 1040
tissue factor pathway inhibitor (TFPI), 349, 733, 1040
gene restoration of, 982
tissue factor plasminogen inhibitor (TFPI), 107

Cambridge Books Online © Cambridge University Press, 2009

tissue plasminogen activator (t-PA), 503, 691, 739, 991
gene restoration of, 981
Toxoplasma gondii, 816–17
transforming growth factor beta (TGFβ), 107, 108, 403, 826, 993
megakaryopoiesis inhibition, 34
role in asthma, 858
role in glomerular diseases, 843–4
transfusion *see* platelet transfusion; red blood cells
transient ischemic attack (TIA), 922, 1045–9
see also stroke
transient receptor potential (Trp) channel proteins, 263, 265–6
trials *see* clinical trial design
trifusal, 955
trigramin, 831
trimucytin, 165
Trypanosoma cruzi, 818
Trypanosoma equiperdum, 818
Trypanosoma musculi, 818
tubulin, 94
see also microtubules
tumour necrosis factor (TNF), 403–4, 991
anti-TNFα antibodies, 912
hemolytic uremic syndrome and, 618
myelodysplastic syndrome and, 535
thrombotic thrombocytopenic purpura and, 618
tumours *see* malignancy
tymus- and activation-regulated chemokine (TARC), 396, 398
tyrosine kinases, 83, 99–100, 281–7, 680
ADP and, 128
calcium and, 225, 265
collagen receptor interactions, 165–7, 169, 283–4
focal adhesion kinase (FAK), 283, 284–5, 286, 287
GTPase interactions, 207, 213
inhibitors, 422
JAK family kinases, 285
phospholipase activation, 241, 246–7, 252–3
platelet–leukocyte adhesive mechanisms, 417–19
Src-family tyrosine kinases, 99, 165–7, 169, 281–3
Syk tyrosine kinase, 87, 99, 246–7, 283–4, 286, 765
Tec family kinases, 285
tyrosine phosphorylation events in human platelets, 286–7
inside–out signalling, 286
outside–in signalling, 286–7
platelet shape change and, 325–6

U46619, 472
ubiquitin, 818
ulcerative colitis, 907
clinical features, 907
etiopathogenesis, 908
see also inflammatory bowel disease
unstable angina, 374, 421, 728, 734, 743
aspirin benefits, 922
mean platelet volume and, 75, 76
platelet shape change and, 329
Upshaw–Schulman syndrome, 610
uremia, 692–4, 837–42
anemia role, 839–40
drug effects, 840
platelet abnormalities, 838
platelet–vessel wall interaction and, 838–9
thrombocytopenia and, 838
thrombotic complications, 840–2
treatment, 693–4, 840, 841
see also dialysis; hemolytic uremic syndrome (HUS)
urokinase, 691
uterus, platelet activating factor and, 886–7, 889–90

variable numbers tandem repeat (VNTR) polymorphism, 467, 763
vascular cell adhesion molecule-1 (VCAM-1), 738
vascular damage, 419–20
Crohn's disease and, 908
see also atherosclerosis; vessel wall injury
vascular endothelial growth factor (VEGF), 107, 403–4, 829, 984
gene transfer, 985
vascular endothelium *see* endothelium
vascular permeability factor (VPF), 107
vascular smooth muscle cells (VSMC)
accumulation of, 978, 980
antiproliferative gene therapy, 982–3
apoptosis promotion, 983
inhibition of VSMC migration, 984
role in restenosis, 982
vasoactive substances *see* endothelium
vasoconstriction, 734
vasodilator-stimulated phosphoprotein (VASP), 296, 298, 932
platelet shape change and, 327
VCAM-1, 394
vein grafts
failure of, 985
gene therapy and, 985–6
implications for gene delivery, 985
prevention of vein graft deterioration, 985
velocardiofacial syndrome (VCFS), 520, 565
venoms, 165
vesicle-associated membrane protein (VAMP), 363
vessel wall injury, 4–5
gene transfer of cyclooxygenase-1 to sites of arterial injury, 981–2
response to, 738–9
see also atherosclerosis; inflammation; vascular damage
viral infection, 807–12
aplastic anemia and, 531
childhood thrombocytopenia and, 562
virus inactivation in platelet concentrates, 810–12
virus–megakaryocyte interactions, 807–9
virus–platelet interactions, 807–9
see also specific viruses
viridans streptococci, infective endocarditis, 784–5

Cambridge Books Online © Cambridge University Press, 2009

vitamin B6, 478
vitamin B12, 478
  deficiency, 533–4
vitamin C, 478
vitamin E, 330, 478
volume increases, 323
  see also mean platelet volume (MPV)
von Willebrand factor (vWF), 5, 10, 179, 307–8
  diagnostic tests, 342
  endothelial dysfunction and, 437–8
  phospholipase C activation, 240
  platelet adhesion and, 307–8, 310, 459, 744, 838
    uremia effects, 839
  platelet surface activation, 48, 53, 113, 183
    GPIb–IX–V complex interaction, 85–6, 94, 179, 181, 183, 941–2
    integrin interaction, 82, 179
  receptor (GPIb–IX–V complex) *see* glycoproteins
  release of, 358
  role in hemostasis, 179, 304–5
    collagen interaction, 307–8
  role in thrombosis, 438, 460
  synthesis, 437
  thrombotic thrombocytopenic purpura and, 611
von Willebrand factor (vWF)-cleaving protease
  assays, 613–15
  deficiency in thrombotic thrombocytopenic purpura, 612–13, 616–17
von Willebrand's disease, 5, 9, 179, 520
  acquired, 696–7
  deficiency, 9, 182–3
  platelet adhesion and, 312–13, 767–8
  platelet-type, 182–3, 520, 565–6, 642
  therapy, 683, 714
  type 2B, 182–3, 520

Waldenström macroglobulinemia, 696
warfarin, 1066
  genetic variability in response, 967–8, 970–2
  glomerular disease treatment, 844
Wilson's disease, 329
Wiskott-Aldrich syndrome (WAS), 98, 214, 312, 515–17, 564, 682
  WAS protein (WASP), 98, 207, 214, 515–17, 564, 682
Wistar Furth (WF) rats, 661–2

X-linked agammaglobulinemia (XLA), 247, 285
X-linked thrombocytopenia, 515–18, 564, 682
  macrothrombocytopenia, 517–18
  microthrombocytopenia, 515–17
xemilofiban, 949

zinc deficiency, 347

Cambridge Books Online © Cambridge University Press, 2009

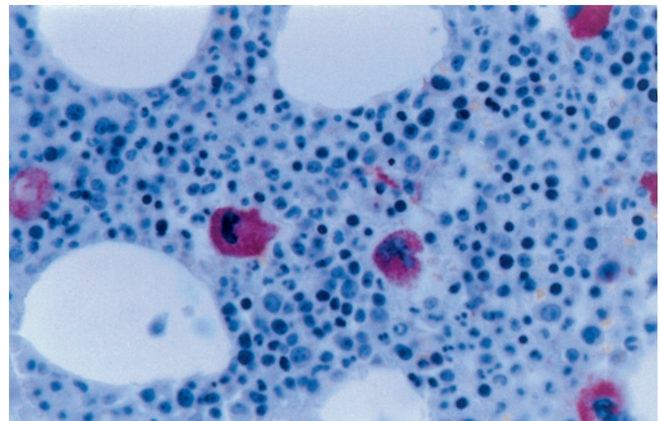


Fig. 2.1. Human bone marrow section stained for fibrinogen by the APAAP technique: MKs specifically stain red. They display a large size and polylobulated nucleus, and are quite seldom in the marrow space compared with the other hematopoietic cell lines. They often stand close to a vascular sinusoid.

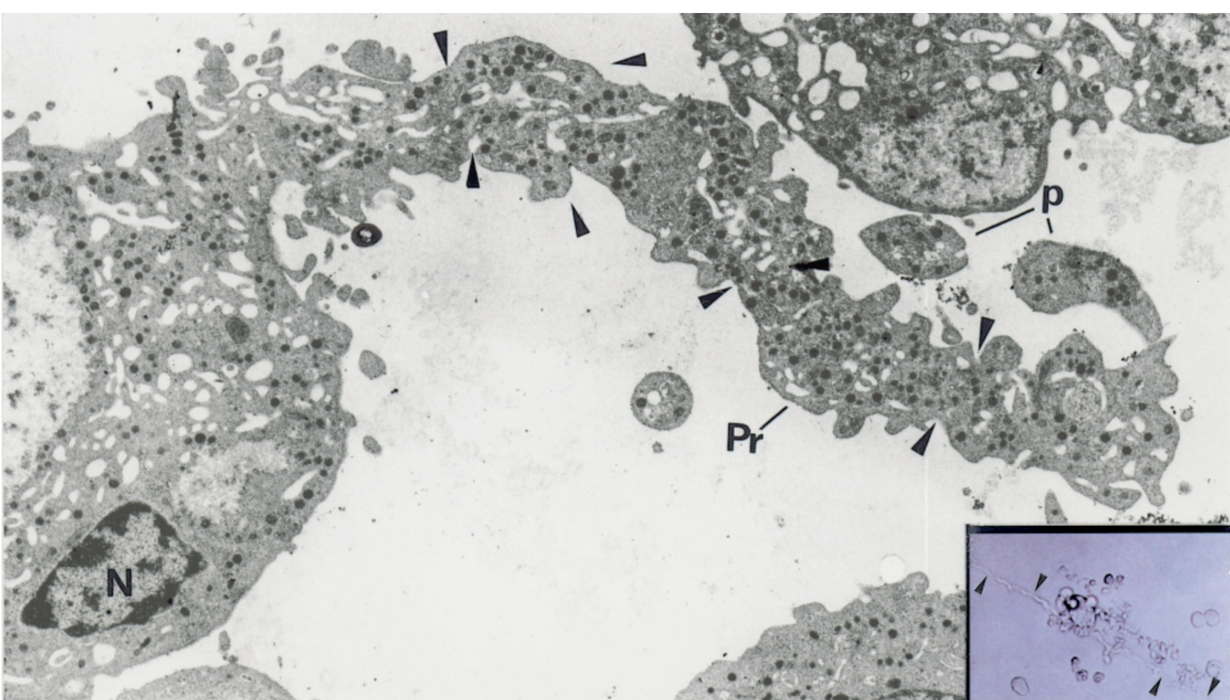


Fig. 2.3. After reaching full maturation, MK cytoplasm extends away from the cell core of the mother cell forming one or several long extensions called proplatelets (pr). Some platelets (p) have detached from its tip. N = nucleus (M × 3354).
Inset: Phase contrast microscopic view of a mature MK extending proplatelets (arrowheads).

Cambridge Books Online © Cambridge University Press, 2009

Platelet shape	Actin	Actin filament ends	Main signals
Discoid	40 % of actin polymerized into filaments	CapZ and adducin cap actin filament barbed ends	$[Ca^{2+}] < 100$ nM ppl's sequestered
Round	40 % of actin polymerized into filaments	Gelsolin severs actin filaments CapZ, adducin and gelsolin are on barbed ends	PLC-mediated hydrolysis of ppl's Cytosolic calcium rises to 0.5-3 μM
Spread	80 % of actin polymerized into filaments	Gelsolin and adducin dissociate from barbed ends Arp2/3 is activated and nucleates actin assembly de novo CapZ associates with barbed ends	ppl's are synthesized: PI_4P up by 80 μM $PI_{4,5}P_2$ up by 60 μM $PI_{3,4}P_2$ up >20 fold $PI_{3,4,5}P_3$ up transiently

Fig. 6.3. The role of calcium and ppIs in the regulation of the platelet actin cytoskeleton. This diagram links platelet shape change during activation with changes in the actin cytoskeleton, proteins that regulate actin filament ends and the primary signals that effect changes in filament ends. The actin filaments in the discoid resting platelet are shown in a mustard-yellow colour, while the capping proteins are shown in pink and purple. Actin monomers that add to the barbed end of filaments in the spread platelet are illustrated as blue subunits.

Cambridge Books Online © Cambridge University Press, 2009

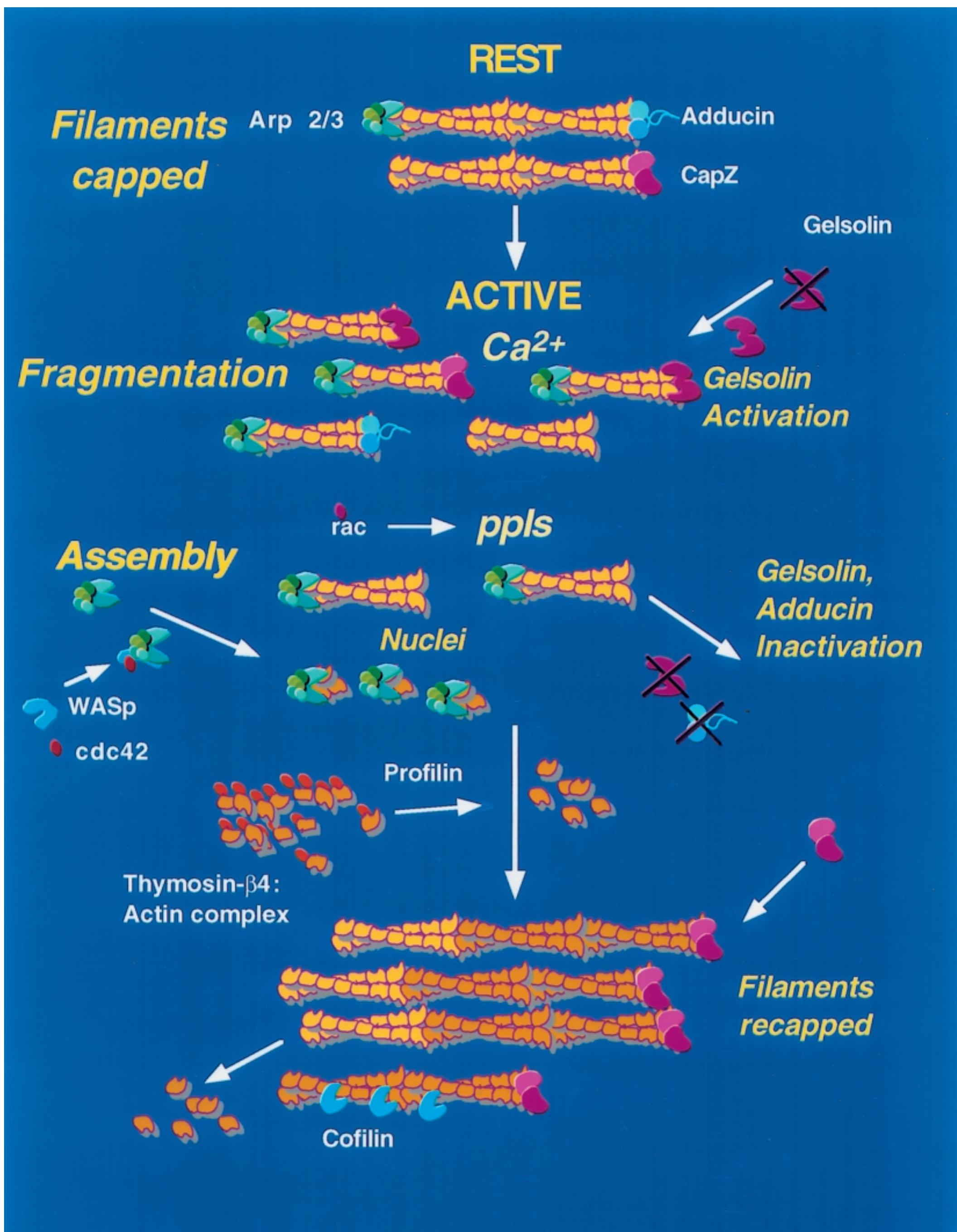


Fig. 6.4. Regulation of platelet actin assembly. The role of known platelet proteins in the actin assembly reaction is shown. In the resting platelet, adducin and CapZ cap actin filament barbed ends. Activation leads to calcium release and the activation of gelsolin. Gelsolin fragments the cortical actin filament network and this event is responsible for the loss of the discoid shape of the resting platelet, which now assumes a more rounded morphology. Polyphosphoinositides are robustly generated at the cytoplasmic surface of the platelet plasma membrane and lead to the initiation of actin assembly onto exposed barbed ends of actin filaments. PpIs serve to both inactivate capping proteins (adducin and gelsolin) and to maximally activate the Arp2/3 complex through the WASp family of proteins. Profilin and thymosin β4, by binding to actin monomers, maintain the large actin pool that drives filament assembly. Actin filaments are subsequently buffered by CapZ, which recaps the filaments, and terminates the assembly reaction. Subsequent actin filament turnover if it occurs in platelets is stimulated by cofilin.

Cambridge Books Online © Cambridge University Press, 2009

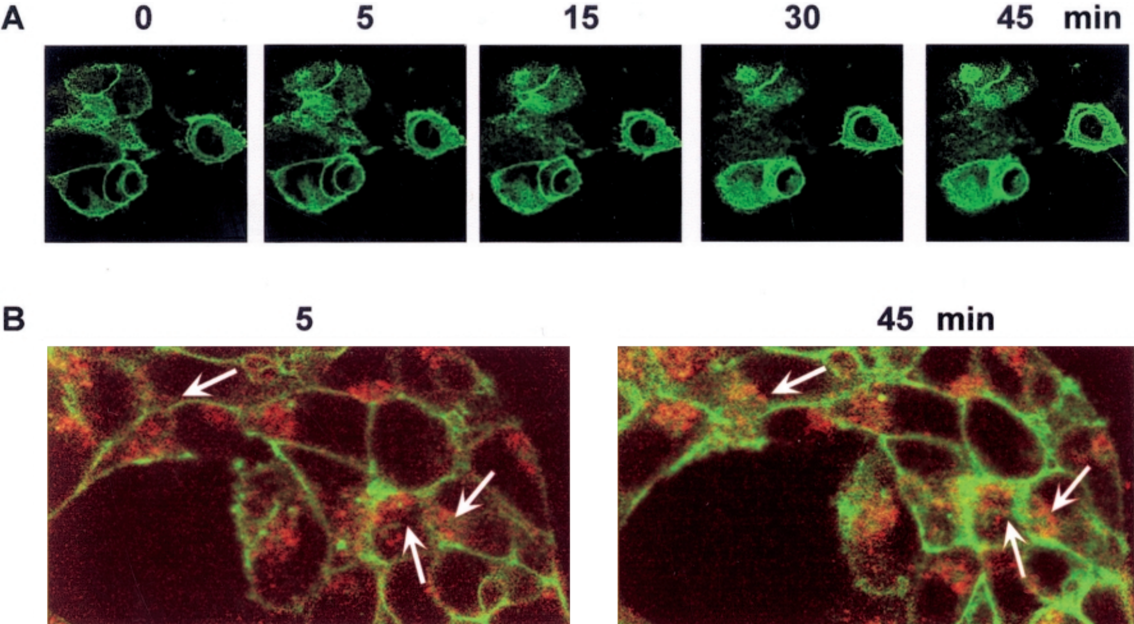
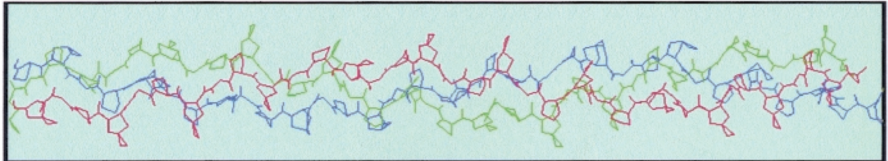


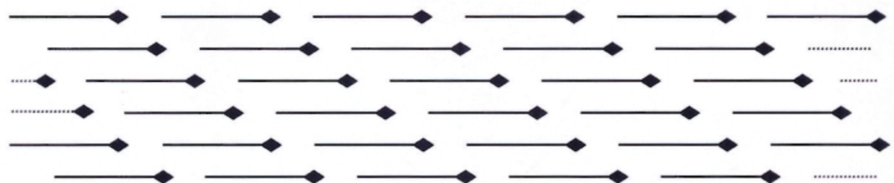
Fig. 10.6. Confocal imaging of hemagglutinine (HA) tagged human IP fused to green fluorescent protein (GFP) (HAhIP–GFP) cells in real time. (*a*) HAhIP–GFP cells were treated with 1 μM iloprost and images acquired at the indicated times. (*b*) HAhIP–GFP (green) cells were preloaded with rhodamine-conjugated transferrin (red) and images acquired at 5 and 45 min after treatment with 1 μM iloprost. Areas of co-localization (yellow) are indicated by the arrows. Data are from one experiment that was repeated with similar results. Reproduced from Smyth et al., 2000[94].

Cambridge Books Online © Cambridge University Press, 2009

Triple-helical collagen monomer



Monomers assemble to form fibril



Native collagen fibre

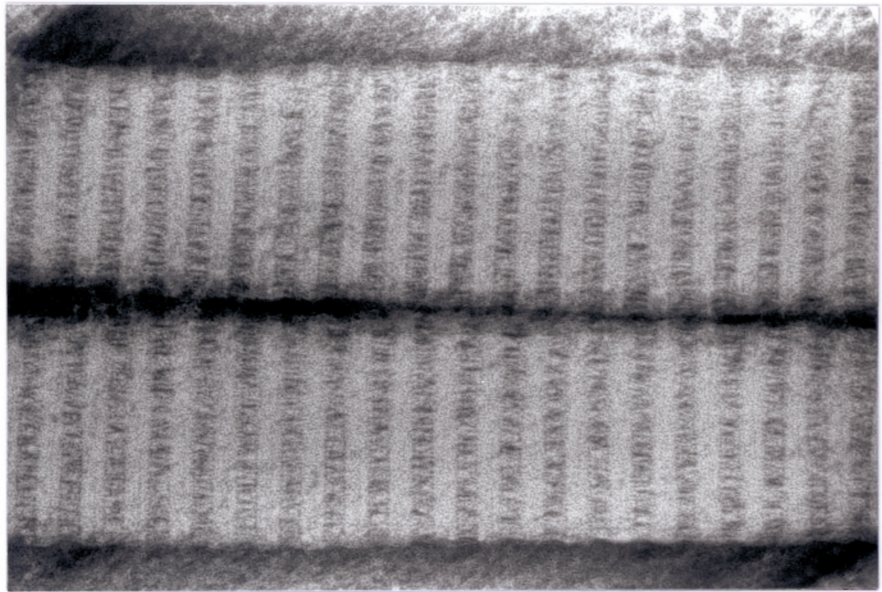


Fig. 11.1. illustrates the hierarchical structure of collagen: the monomer is represented using the crystal coordinates of $[GPO]_{12}$, obtained from 2clg.pdb, with its individual alpha chains shown in different colours, and the proline and hydroxyproline residues protruding into the external medium. The tropocollagen monomers assemble head-to-tail in a quarter-staggered array into fibrils, and their organization into fibres is clearly visualized by transmission electron microscopy. ◆ indicates the N-terminus of the tropocollagen molecule. (Transmission electron microscopy, courtesy of M. Hess, University of Helsinki.)

Cambridge Books Online © Cambridge University Press, 2009

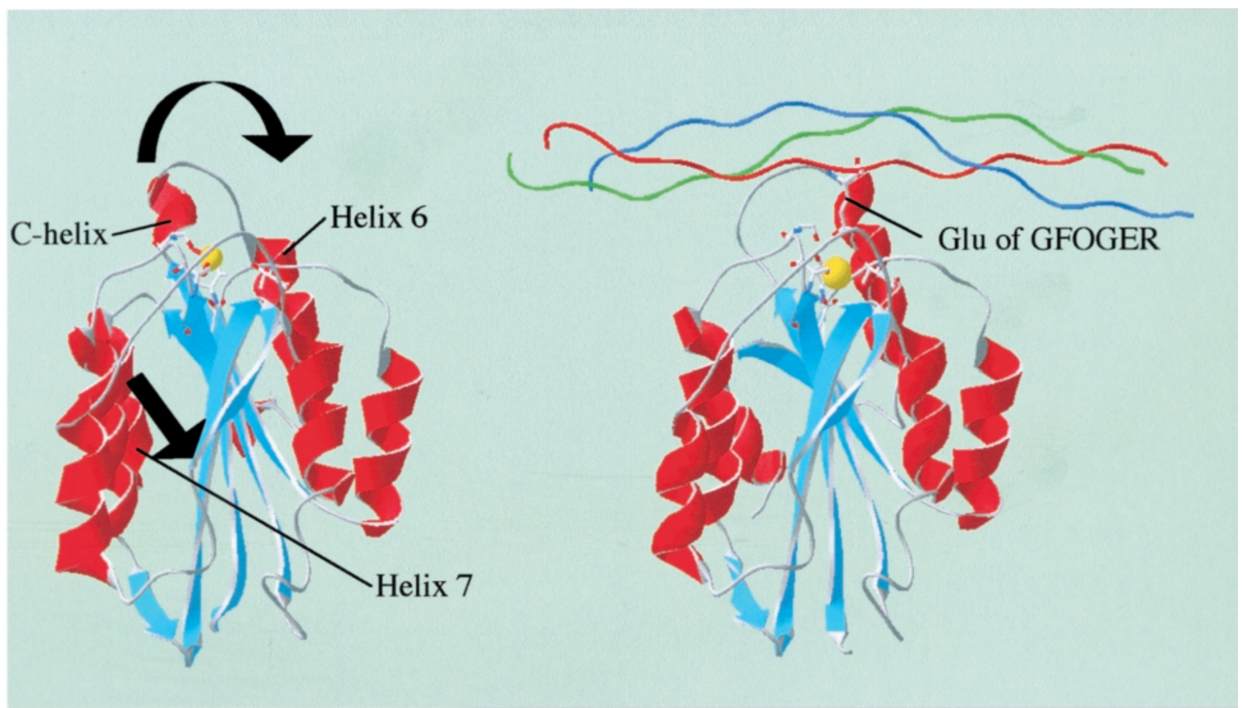


Fig. 11.2. Shows the differing conformation of the free integrin $\alpha 2$ I-domain compared with that bound by collagen. As indicated by the black arrows, the resting I-domain moves upon ligation by the collagen- like peptide. The C-helix unwinds and is added to the top of helix 6. Helix 7 moves axially towards the base of the I-domain. Note that the I-domain construct contains a truncated helix 7, the native form of which, in the active conformation, would protrude from the I-domain and alter its relationship with the rest of the $\alpha 2$ subunit. The metal ion in the MIDAS is shown as a yellow sphere. Crystal coordinates, free I domain, 1aox,.pdb; peptide-I-domain complex, 1dzi.pdb. (Molecular graphics courtesy of Dr D. Tuckwell, University of Manchester.)

Cambridge Books Online © Cambridge University Press, 2009

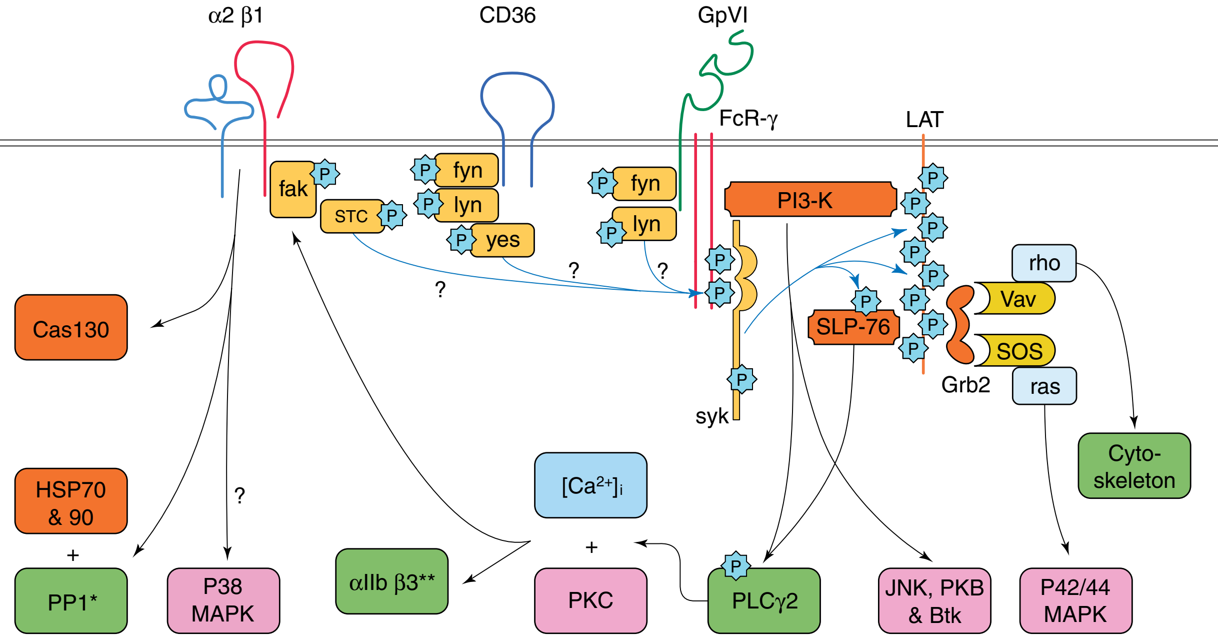


Fig. 11.4. Depicts proposed signalling pathways from the major collagen receptors, $\alpha 2\beta 1$, CD36 and GpVI. Phosphorylation events are shown using blue arrows, and other signalling pathways in black. Question marks indicate possible signalling pathways, lacking specific detail at present. Signalling molecules are defined in the text. Tyrosine kinases are coded *beige*, serine/threonine kinases *pink*, adapter proteins in *orange*. Tyrosine phosphorylation is indicated by stars on the relevant proteins. Other collagen receptors and possible signals arising from the GpIb/IX/V axis are omitted for clarity.

Cambridge Books Online © Cambridge University Press, 2009

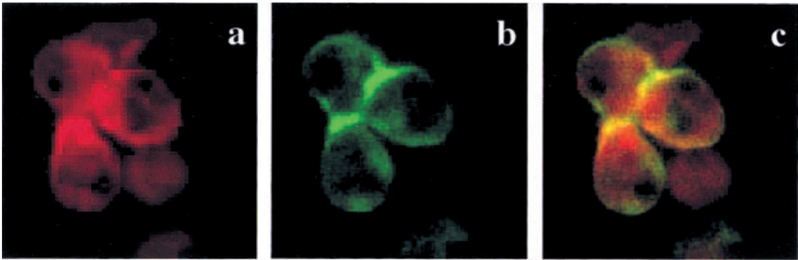


Fig. 28.4. Formation of Mac-1 clusters and colocalization with F-actin patches in PMN leukocytes at the site of cell–cell contact. Confocal laser scanning microscopy of PMN, preincubated with an anti-PSGL-1 antibody, stained with FITC-conjugated anti-CD11b antibody and rodamine-phalloidin: (*a*) and (*b*) show F-actin (red) and CD11b (green) staining, respectively. In the overlay (*c*), the yellow colour represents colocalization of the two stainings. (For more details[141].)

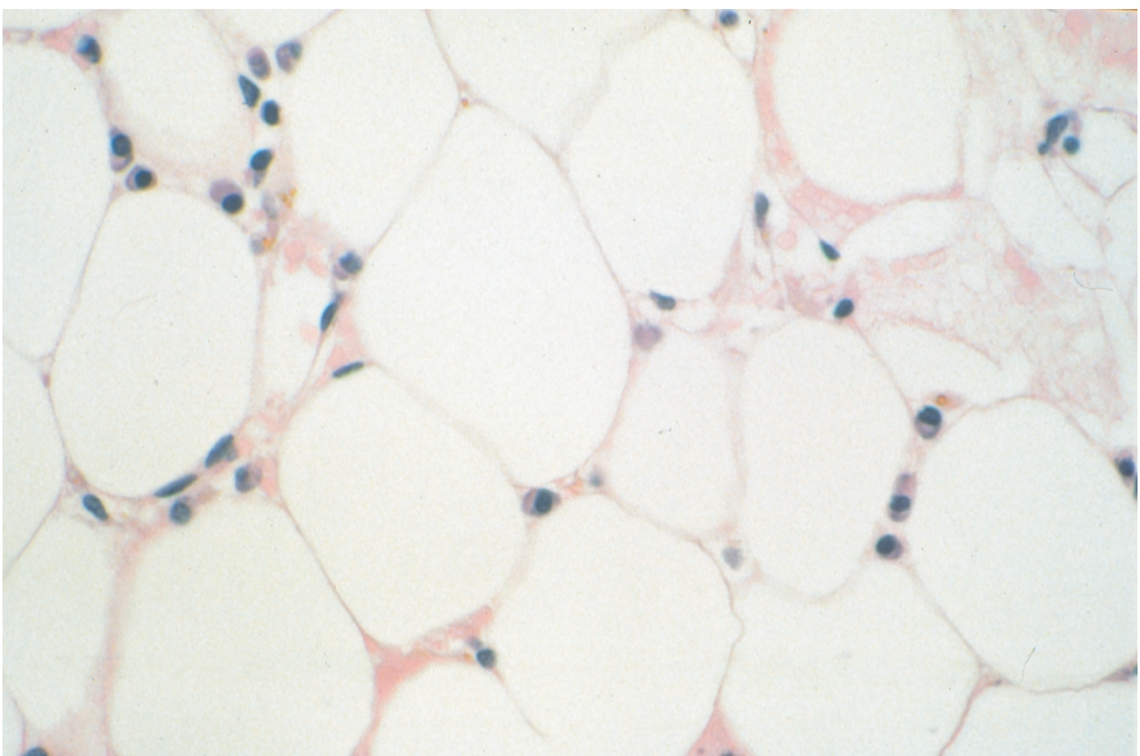


Fig. 35.2. A bone marrow trephine biopsy of a patient (male, 25 years of age) with aplastic anemia. It shows predominantly adipose spaces and stromal cells but very few hematopoietic cells.

Cambridge Books Online © Cambridge University Press, 2009

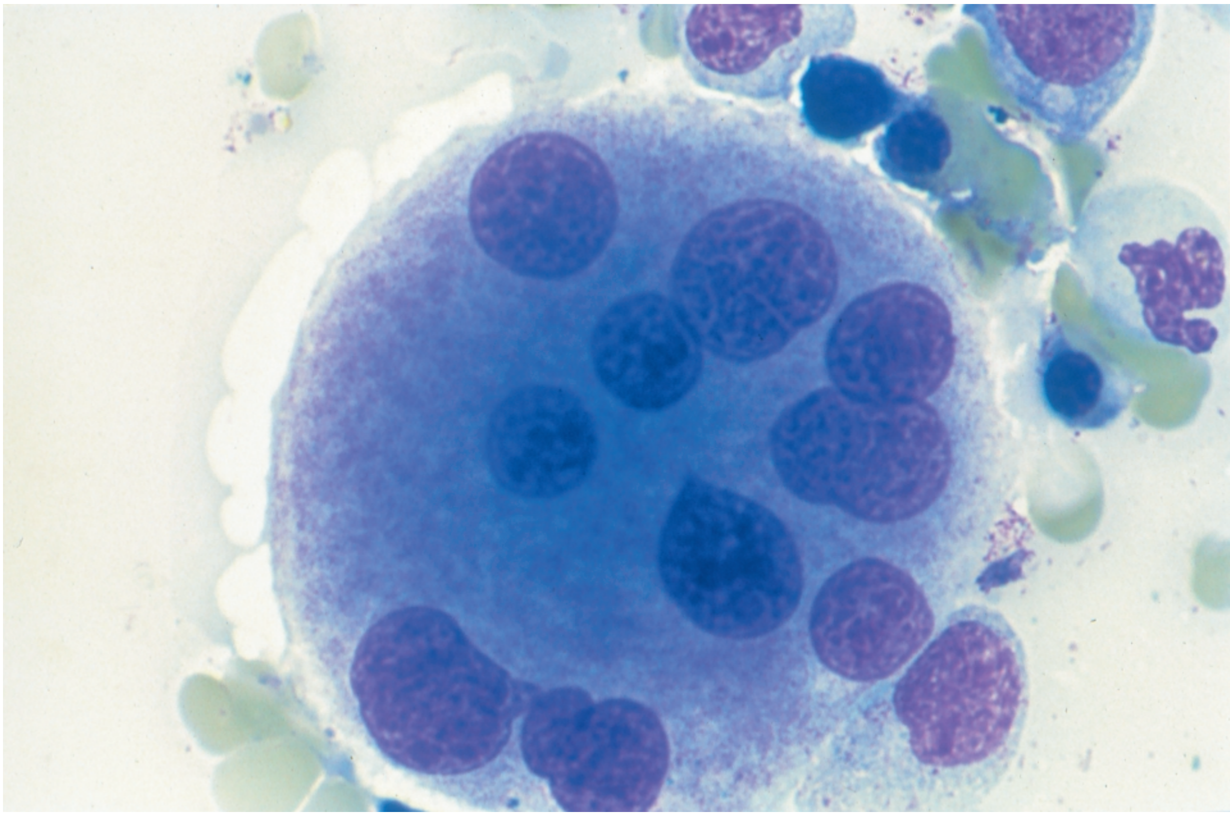


Fig. 35.3. This shows an abnormal megakaryocyte with dysplastic changes in a patient with HIV-1 infection and thrombocytopenia. It is a huge cell with nuclear fragmentation.

Cambridge Books Online © Cambridge University Press, 2009

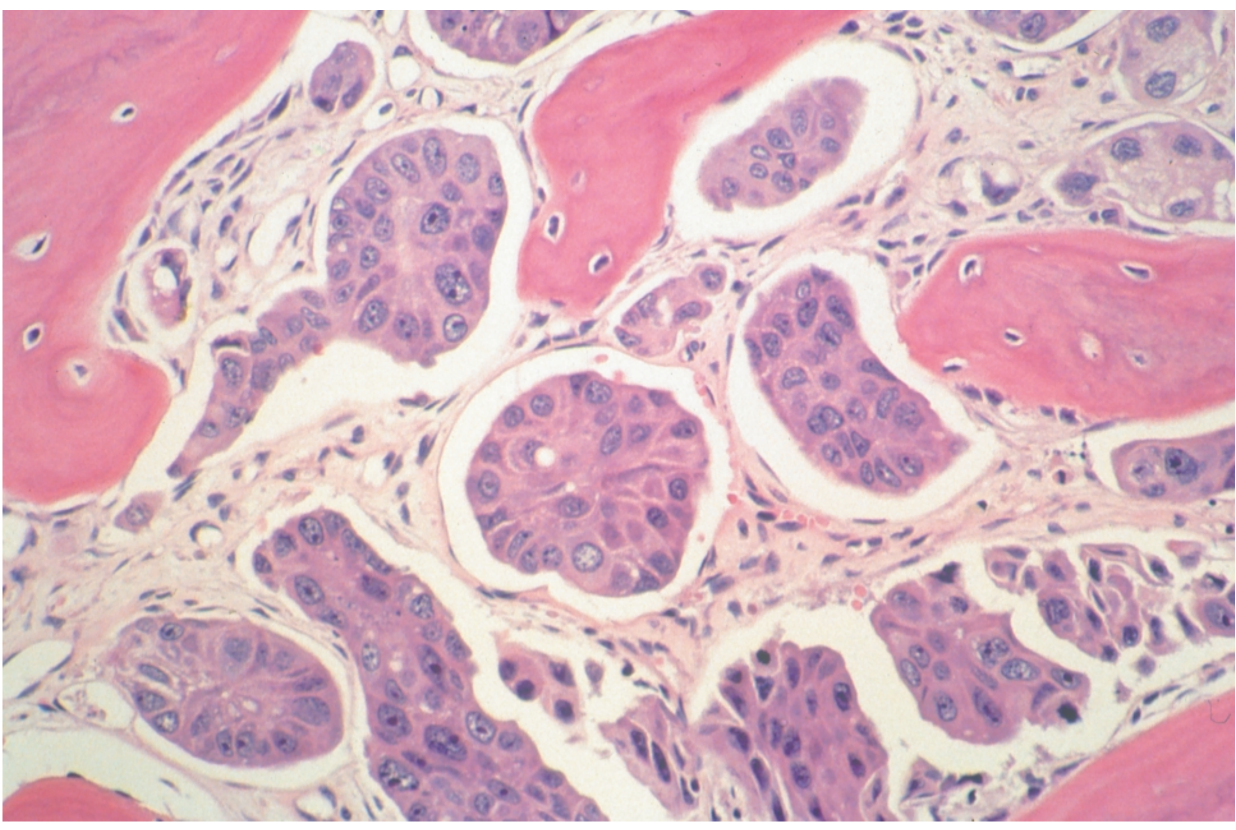


Fig. 35.4. A bone marrow trephine biopsy of a patient with metastatic breast cancer showing bone marrow infiltration by malignant cells. The cancer cells are arranged in a gland-like formation (acini).

Cambridge Books Online © Cambridge University Press, 2009

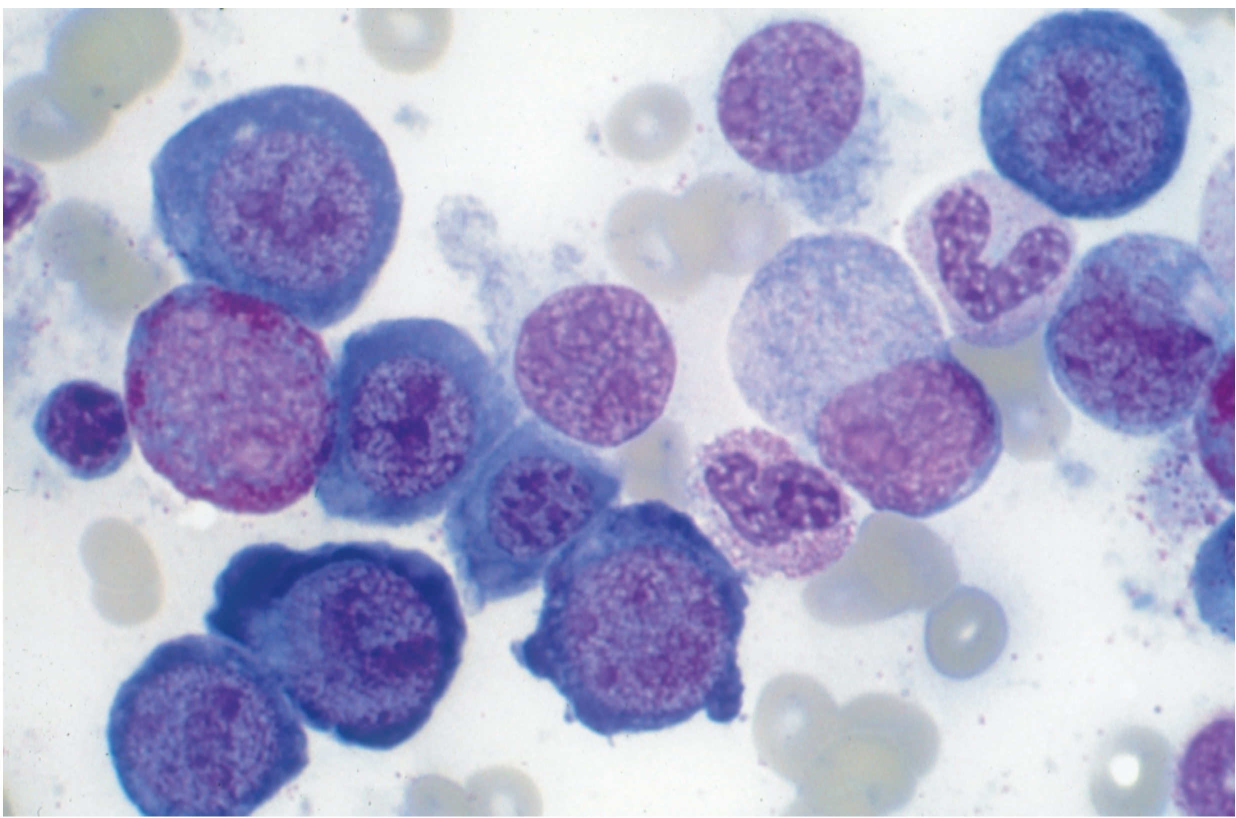


Fig. 35.5. A bone marrow smear of a patient (female, aged 76) with pernicious anemia. It shows florid megaloblastic changes with many megaloblasts and a giant band form.

Cambridge Books Online © Cambridge University Press, 2009

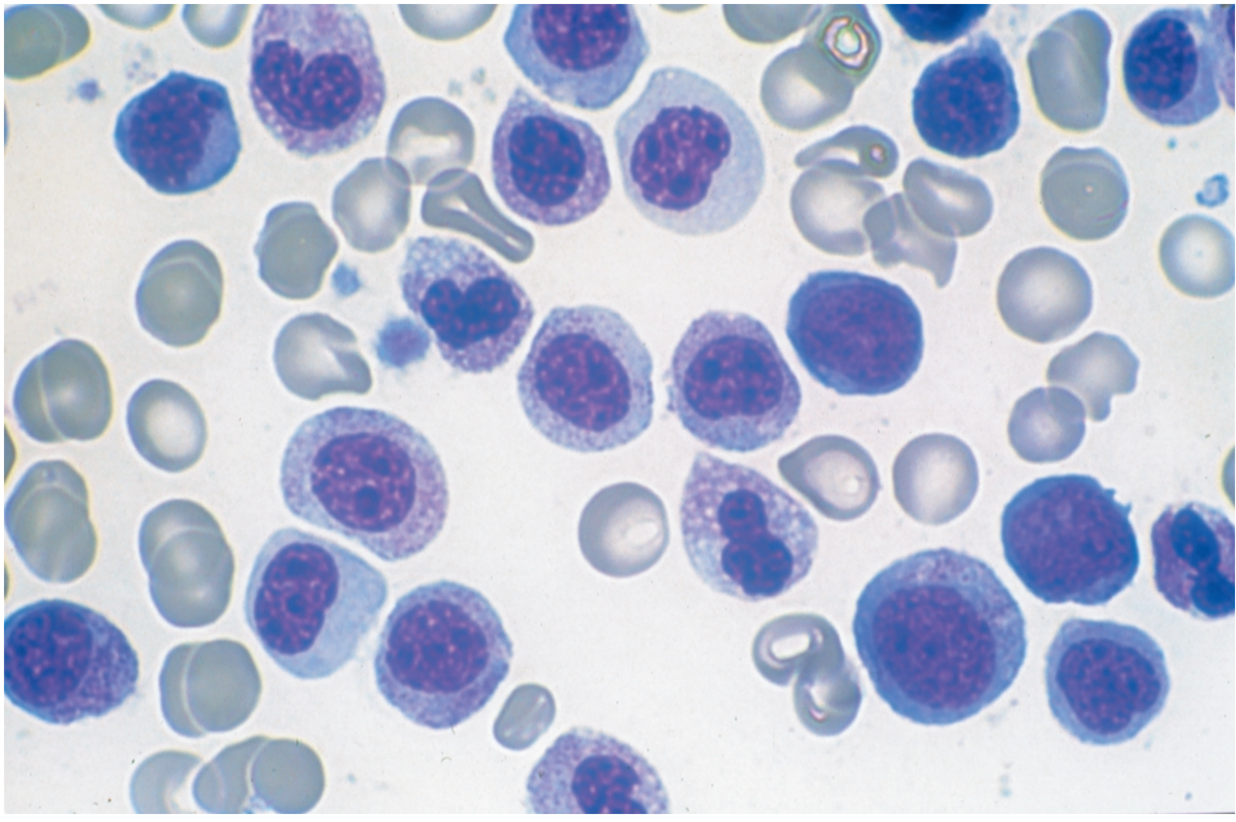


Fig. 35.6. A bone marrow smear of a patient (male, aged 64) who had myelodysplastic syndrome. It shows dysplastic changes in granulocyte precursors, namely hypogranular forms and Pelger–Huet anomaly.

Cambridge Books Online © Cambridge University Press, 2009

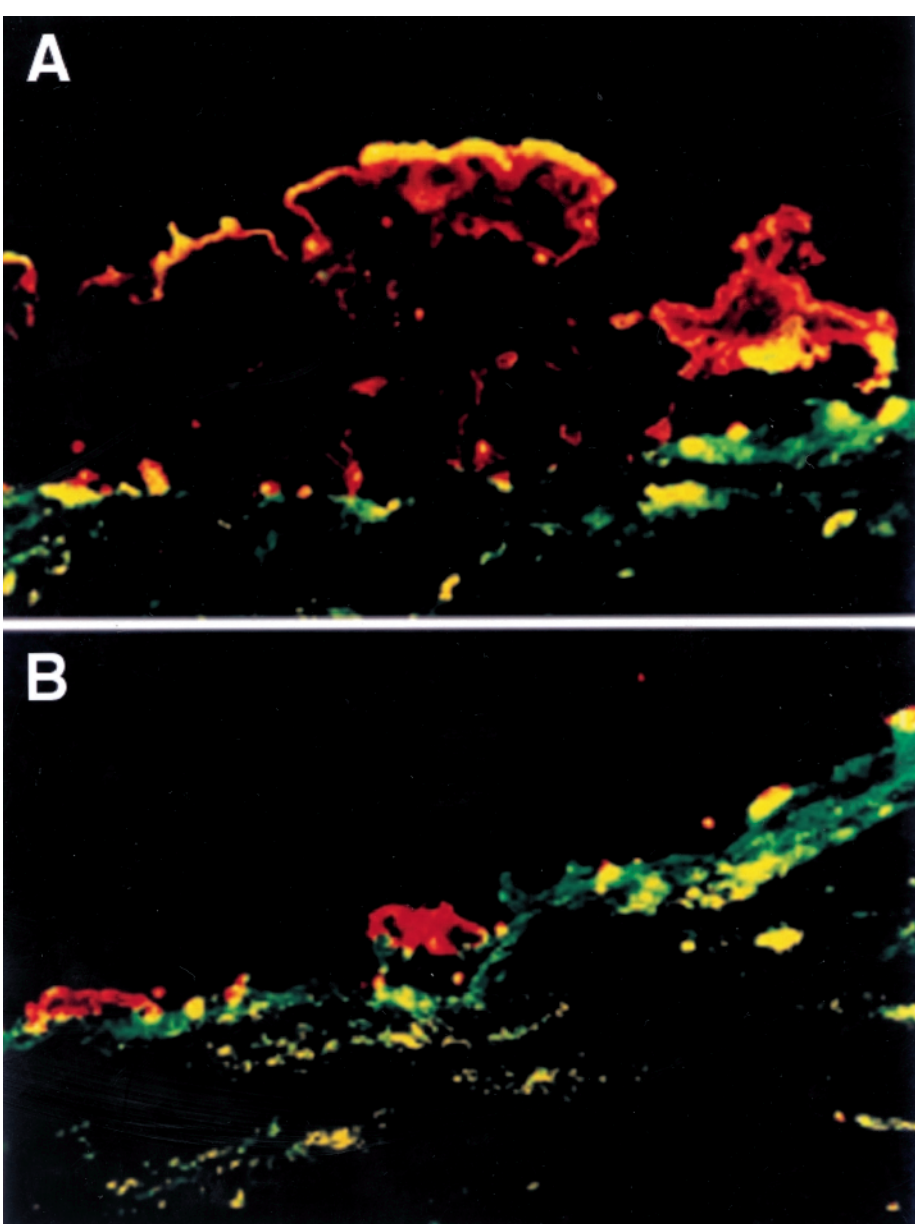


Fig. 47.5. Effects of TFPI on thrombogenicity of disrupted lipid-rich human atherosclerotic lesions: Control (*a*) and TFPI-treated (*b*). Fibrinogen deposition is shown as green, platelet deposition as red, and their colocalization as orange. Note the significant antithrombotic effect of rTFPI on deposition of platelet and fibrin(ogen) deposition.

Cambridge Books Online © Cambridge University Press, 2009

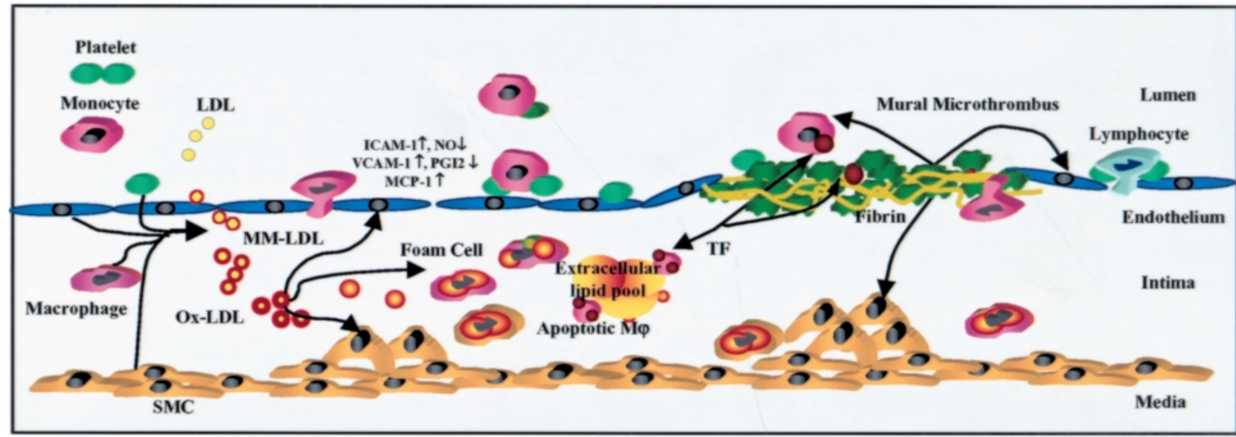


Fig. 48.2. Endothelial cells, platelets, smooth muscle cells and macrophages mediate oxidative modifications of LDL. MM-LDL activates endothelial cells and promotes smooth muscle cell migration and proliferation. Uptake of oxidized lipoproteins by macrophages and smooth muscle cells generates foam cells. Activated platelets adhere to, and activate, endothelium and facilitate monocyte adhesion. Endocytosis of platelet-derived vesicles contributes to foam cell formation. Endothelial erosion initiates platelet adhesion and aggregation and fibrin deposition on the subendothelial matrix. Tissue factor is closely associated with macrophages in the lipid core. Propagation of a thrombus may also depend on blood-borne tissue factor. Platelets release a number of growth factors propagating smooth muscle cell proliferation. Re-endothelialization and cap formation by smooth muscle cells incorporates the mural thrombus into the expanded lesion. (ICAM-1: intercellular adhesion molecule-1, M*ψ*: macrophage, MCP-1: monocyte chemotactic protein-1, MM-LDL: Minimally modified LDL, NO: nitric oxide, ox-LDL: oxidized LDL, PGI_2: prostacyclin, SMC: smooth muscle cell, TF: tissue factor, VCAM-1: vascular cell adhesion molecule-1.)

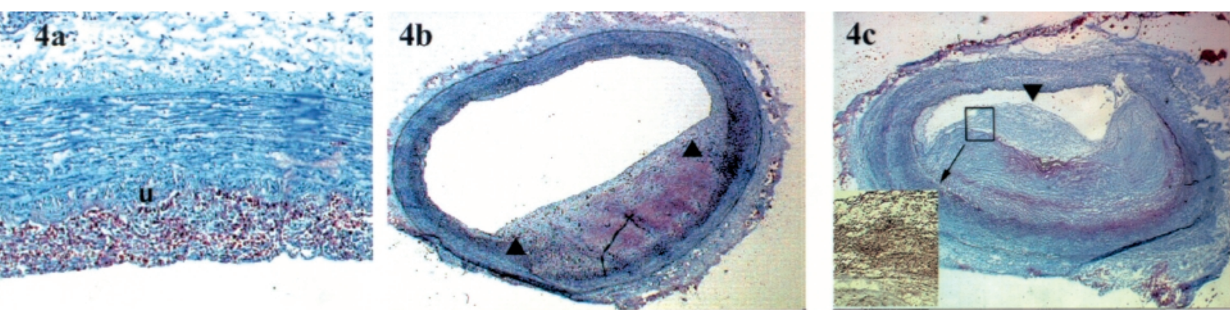


Fig. 48.4. (*a*) Oil Red O staining of a fatty streak (Stary type II lesion). The lesion is rich in foam cells. Smooth muscle cells (▶) migrate towards the intima.(*b*) Oil Red O staining of a fibroatheroma (Stary type IV–V). The eccentric lesion consists of a large lipid core with a rather thin fibrous cap. Plaque rupture occurs most frequently at the shoulder regions of the plaque (▲). (*c*) Oil Red O staining of a complicated lesion (Stary type VI). The layered aspect and the organized thrombus (▼) are hallmarks of healed disruption. Immunostaining (➔) for fibrin shows fibrin at the basis of the most recently formed cap.

Cambridge Books Online © Cambridge University Press, 2009

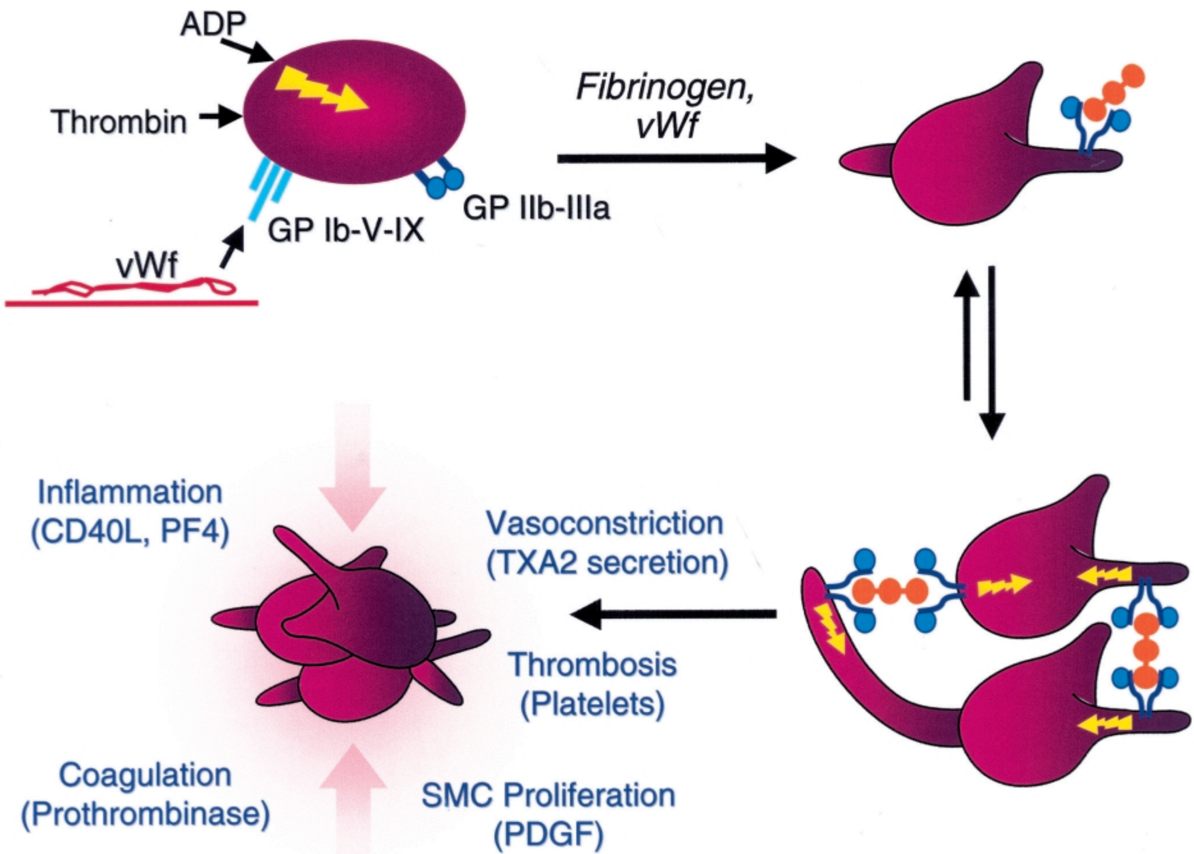
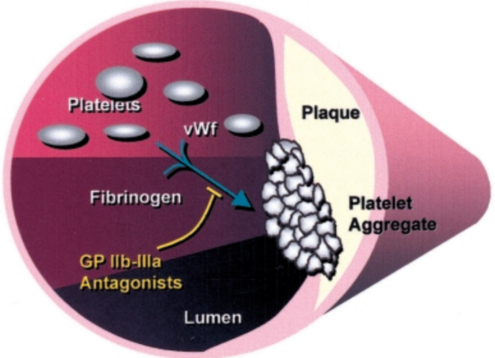
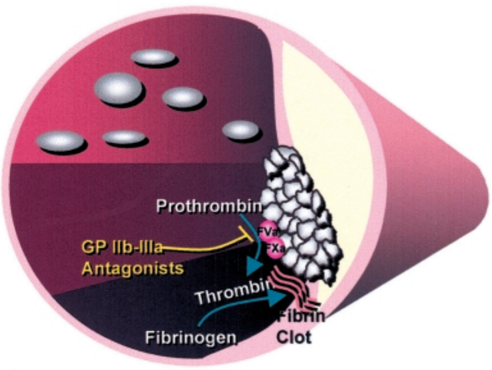


Fig. 63.1. Schematic illustrating the role of GP IIb–IIIa in platelet aggregation. On resting, discoid platelets the GP IIb–IIIa is present in an 'inactive' conformation and is unable to bind soluble fibrinogen. Upon activation by classical agonists such as collagen, thrombin or adenosine diphosphate (ADP) the platelet undergoes a shape change and the GP IIb–IIIa is activated such that it can now bind its soluble ligands, fibrinogen and von Willebrand factor (vWf). Platelet aggregation is mediated by the bivalent fibrinogen forming bridges between GP IIb–IIIa molecules on the surfaces of neighbouring, activated platelets.

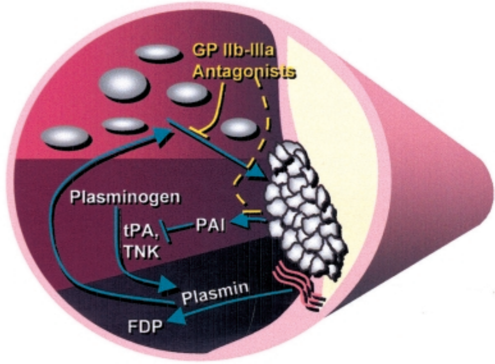
Cambridge Books Online © Cambridge University Press, 2009



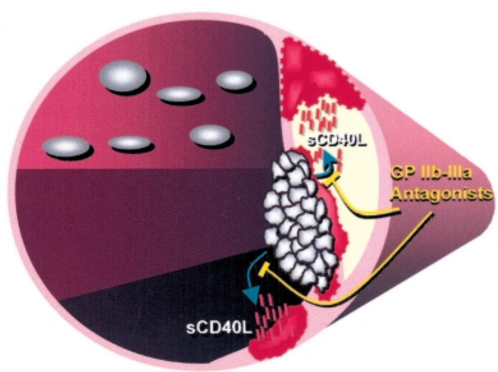
A. Thrombosis



B. Coagulation



C. Fibrinolysis



D. Inflammation

Fig. 63.2. Diagrams depict the multiple benefits of GP IIb–IIIa antagonists. (*a*) GP IIb–IIIa antagonists block platelet aggregation and recruitment to prevent thrombosis. (*b*) The prothrombinase complex (FVa, FXa) is predominantly formed on the surface of aggregated platelets. The anticoagulant activity of GP IIb–IIIa antagonists can be attributed to their antiaggregatory activity. (*c*) GP IIb–IIIa antagonists inhibit platelet aggregation induced by plasmin and thrombin which are generated during thrombolysis. These antagonists also inhibit the aggregation-induced release of PAI-1, an inhibitor of fibrinolysis. The apparent 'fibrinolytic' activities of GP IIb–IIIa antagonists can be attributed to these synergistic activities with fibrinolytic agents. (*d*) Platelet aggregation induces the expression and release of the potent proinflammatory protein CD40L. Inhibition of platelet adhesion to vascular cells and sCD40L release from platelets by GP IIb–IIIa antagonists may allow this class of drugs to be anti-inflammatory.

Cambridge Books Online © Cambridge University Press, 2009